Induction of Rhinacanthin Formation in *Rhinacanthus nasutus* (L.) Kurz Tissue Cultures

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ชื่อวิทยานิพนธ์ การเหนี่ยวนำการสร้างสารไรนาแคนตินในเนื้อเยื่อเพาะเลี้ยงของต้นทองพันชั่ง
ผู้เขียน นายวิโรจน์ มีรุ่งเรือง
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บทคัดย่อ

ในการสร้างเซลล์เพาะเลี้ยงของทองพันชั่งจากคัลลัสที่ได้จากการเพาะเลี้ยงใบอ่อนของต้นทองพันชั่ง โดยเพาะเลี้ยงเซลล์ของทองพันชั่งในอาหารเหลวสูตร B5 เสริมด้วยฮอร์โมน BA 2.0 มก./ลิตร และ IBA 0.5 มก./ลิตร ทำการถ่ายเซลล์ลงในอาหารใหม่สูตรเดิมทุก 4 สัปดาห์ จากการตรวจสอบการสร้างสาร rhinacanthin ด้วยวิธี HPLC พบว่าเซลล์เพาะเลี้ยงที่ได้ไม่มีการสร้างสาร rhinacanthin เทคนิคการปรับปรุงสูตรอาหารและเทคนิค elicitation ได้ถูกนำมาใช้เพื่อเหนี่ยวนำการสร้างสาร rhinacanthin แต่พบว่าทั้งสองเทคนิคนี้ไม่สามารถเหนี่ยวนำให้เซลล์เพาะเลี้ยงของทองพันชั่งสร้างสาร rhinacanthin ได้ ซึ่งผลที่ได้นี้แสดงให้เห็นว่าเซลล์ที่เกิดสาร dedifferentiate แล้วจะสูญเสียความสามารถในการสร้างสาร rhinacanthin ต่อมาได้ทำการสร้างรากเพาะเลี้ยงของทองพันชั่งในสูตรอาหาร B5 เสริมด้วย IBA 0.1 มก./ลิตร และน้ำตาล 20 กรัม/ลิตร และได้ทำการศึกษาผลของ explant (explant ที่ได้จากใบทั้งใบและใบที่ถูกตัดขอบทั้งสี่ด้าน) และแสงต่อการเหนี่ยวนำให้เกิดรากและการสร้างสาร rhinacanthin จากผลการทดลองแสดงให้เห็นว่า รากที่เกิดจาก explant ที่ได้จากใบทั้งใบมีจำนวนมากกว่ารากที่เกิดจากใบที่ถูกตัดขอบทั้งสี่ด้านถึง 10 เท่า จากการปรับปรุงสูตรอาหารพบว่า สูตรอาหาร MS ที่เสริมด้วย IBA 3.0 มก./ลิตร และน้ำตาล 30 กรัม/ลิตร เหมาะสำหรับการเหนี่ยวนำให้เกิดการสร้างรากของทองพันชั่ง อย่างไรก็ตาม รากเพาะเลี้ยงที่ได้เมื่อนำมาเลี้ยงในอาหารเหลว MS สูตรเดิม กลับพบว่าสามารถสร้างสารได้เพียง rhinacanthin-C ในปริมาณที่น้อยมาก (0.03 ± 0.001 มก./กรัม น้ำหนักแห้ง) แต่อย่างไรก็ตาม เมื่อเลี้ยงโดยใช้อาหารกึ่งแข็งที่เติมวุ้นปริมาณ 4 กรัม/ลิตร ในอาหารสูตรเดิมพบว่า รากเพาะเลี้ยงสามารถสร้างสาร rhinacanthin-C (0.72 ± 0.008 มก./กรัม น้ำหนักแห้ง) และ rhinacanthin-D (0.02 ± 0.000 มก./กรัม น้ำหนักแห้ง) ได้ การศึกษาความสัมพันธ์ระหว่างระยะเวลากับการเจริญเติบโตของรากเพาะเลี้ยงทางพันชั่ง แสดงให้เห็นว่า รากเพาะเลี้ยงมีระยะ lag phase 10 วัน และระยะ linear phase 15 วัน ก่อนที่จะเข้าสู่ระยะ stationary phase และพบว่ามีการสร้างสาร rhinacanthin เพิ่มขึ้น โดยมีปริมาณ rhinacanthin สะสมมากที่สุดในระยะ linear phase (15 วัน) ของวงจรการเจริญเติบโต จากความสำเร็จในการเหนี่ยวนำ

การสร้างสาร rhinacanthin-C และ rhinacanthin-D ของรากเพาะเลี้ยงทองพันชั่งสามารถนำไปใช้เป็นแบบในการศึกษาทั้งทางด้านชีวสังเคราะห์และทางด้านเทคโนโลยีชีวภาพของสารออกฤทธิ์ทางชีวภาพที่สำคัญกลุ่มนี้

Thesis Title Induction of Rhinacanthin Formation in *Rhinacanthus nasutus* (L.) Kurz Tissue Cultures

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ABSTRACT

Cell suspension cultures of *R. nasutus* were established from their callus cultures initiated from the young leaf explants of *R. nasutus*. The cell suspension cultures were maintained in liquid B5 medium supplemented with 2.0 mg/l BA and 0.5 mg/l IBA with periodic subculture into the same liquid B5 medium at 4-week intervals. Analysis by HPLC showed that the cell suspension cultures did not accumulate rhinacanthin. Medium manipulation and elicitation techniques were used to induce their rhinacanthin production. However, neither of them could induce the rhinacanthin formation. These results suggested that dedifferentiation of *R. nasutus* cells causes in loss of rhinacanthin production potential. Subsequently, root cultures of *R. nasutus* were established by using solid B5 medium supplemented with 0.1 mg/l IBA and 20 g/l sucrose. The effects of explants (whole leaf explants and the four-side excised leaf explants) and light on root and rhinacanthin formation were investigated. The results showed that the root formation from the whole leaf explants was 10 times higher than that from the four-side excised leaf explants. Medium manipulation found that MS medium supplemented with 3.0 mg/l IBA and 30 g/l sucrose was the most suitable for induction of the root formation. However, the obtained root cultures produced only rhinacanthin-C in very low amount (0.03 mg/g DW) when they were transferred into the same MS liquid medium. With semisolid medium (4 g/l agar) of the same MS composition, however, the root cultures appeared to produce both of rhinacanthin-C and rhinacanthin-D with the content of 0.72 and 0.02 mg/g DW, respectively. Study on the time-course of growth of *R. nasutus* root cultures showed the root cultures in a lag phase of 10 days and linear phase of 15 days before entering to the stationary phase. Rhinacanthin production was found to increase with highest its rhinacanthin accumulation at the linear phase (day 15) of the

growth cycle. The success in the induction of rhinacanthin-C and rhinacanthin-D production in the root cultures of *R. nasutus* will allow us to use it as a model for both biosynthetic and biotechnological studies of this important group of bioactive compounds.

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Finally, I am grateful to my family for their support, entirely care, and love. The usefulness of this thesis, I dedicate to my parent and all the teachers who have taught me since my childhood.

Wirod Meerungrueang

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LIST OF ABBREVIATIONS

DW	dry weight
ED_{50}	median effective dose
g	gram
HPLC	high pressure liquid chromatography
IC_{50}	inhibitory concentration at 50% of tested
subject	
L	liters
mg/l	milligrams per liter
MIC	minimum inhibitory concentration
mM	millimolar
ml	milliliter
ng	nanogram
μg	microgram
μM	micromolar

CHAPTER 1

INTRODUCTION

A typical feature of plants is the production and accumulation of secondary metabolites. Secondary metabolites are compounds biosynthetically derived from primary metabolites. They are not essential for energy metabolism and life. These products appear to be important in the interactions between the plant and its environment (Wink, 1988; Harborne, 1993; Wink, 2006). Their major functions include pollinator attractants, represent chemical adaptations to environmental stresses, or serve as chemical defenses against microorganisms, insects and higher predators, and even other plants (Levin, 1976; Swain, 1977; Harbone, 1982). Therefore, secondary metabolites are of major interest because of their different functions and their impressive biological activities ranging from antimicrobial, antibiotic, insecticidal, hormonal properties to highly important pharmacological and pharmaceutical activities (Stockigt *et al*., 1995).

Rhinacanthus nasutus is a plant in Acanthaceae family. It is so called in Thai “Thong phan chang” or “Yaa man kai”. It widely distributes in Southeast Asia, South China and India (Farnsworth and Bunyapraphatsara, 1992). In Thailand, the Thai foundation health committee, Ministry of public health has recommended the leaves and roots of *R. nasutus* for the treatment of tinea vesicolor and ringworm (Farnsworth and Bunyapraphatsara, 1992). The groups of compounds commonly found in *R. nasutus* were naphthoquinone, lignan, anthraquinone, benzoquinone, quinol, triterpenoid, sterol, benzenoid, coumarin, flavonoid and amide. The most interest compounds were naphthoquinones, including rhinacanthin-A, -B, -C, -D, -G, -H, -I, -J, -K, -L, -M, -N, -O, -P, -Q (Wu *et al*., 1995; Wu *et al*., 1998b) and rhinacanthone (Kodama *et al*., 1993; Kuwahara *et al*., 1995). These compounds were found in both leaves and roots of *R. nasutus*. Moreover, it has been reported that the rhinacanthins showed interesting pharmacological activities such as antifungal (Wu *et al*., 1998b, Panichayupakaranant *et al*., 2000; 2003, Kongchai and Panichayupakaranat, 2002), antiviral (Kernan *et al*., 1997), antitumour (Thirumurugan *et al*., 2000), anti-platelet aggregation (Wu *et al*., 1998b) and antibacterial (Sattar *et al*., 2004). It has been reported that harvesting periods affect the rhinacanthin content or quality of *R. nasutus* raw materials (Panichayupakaranant *et al*., 2006). Thus, plant tissue cultures are attractive as an alternative source to whole plants for the production of high-value secondary metabolites (flavors, fragrances, and pharmaceuticals) and improve the productivity of plant cell cultures. When compared to traditional agricultural growth, plant tissue culture offers a number of year-round, continuous productions of secondary metabolites under highly controlled conditions (Wink, 2003). Many strategies have been developed to improve the productivity of plant cell cultures such as medium optimization, elicitation, cell immobilization, cell line selection, precursor addition, hairy root cultures, genetic transformation, metabolic engineering and integrated bioreactor engineering (Dornenburg and Knorr, 1995; Abdullah *et al*., 2005).

In this study, *R. nasutus* tissue cultures were established. Induction of rhinacanthin formation using medium manipulation and elicitation techniques was also examined. Recently, the study on rhinacanthin production by *R. nasutus in vitro* cultures is rarely reported. There was only one report on establishment of *R. nasutus* shoot cultures and their rhinacanthin production (ภาคภูมิ พาณิชยูปการนันท์, 2540). However, the shoot cultures have a limitation for further applications when compared to cell suspension cultures due to there growth rate and homogeneity. We therefore studied on the establishment of *R. nasutus* cell suspension cultures and induction of rhinacanthin formation in the cell cultures using medium manipulation and elicitation techniques. In addition, the establishment of the root cultures of *R. nasutus* and determination of their rhinacanthin production were also examined. The obtained tissue cultures may be used as an alternative source of rhinacanthins as well as a material for their biosynthetic studies.

The objectives of the present study were therefore as follow:

1. To induce rhinacanthin accumulation in *R. nasutus* tissue cultures by medium manipulation and elicitation techniques
2. To study on time course of growth and rhinacanthin production in *R. nasutus* tissue cultures

CHAPTER 2

LETERATURE REVIEW

2.1 Botanical aspect of *R. nasutus* (L.) Kurz

Rhinacanthus nasutus (L.) Kurz (*Rhinacanthus communis* Nees) is a plant in Acanthaceae family. It is so called in Thai "Thong phan chang" or "Yaa man kai". It widely distributes in Southeast Asia, South China and India (Farnsworth and Bunyapraphatsara, 1992).

R. nasutus is a small shrub with 70-200 height. The stems of this plant are erect and branched. The leaves are simple and opposite, the shape of the leaves is lanceolate with 2.5 - 5 cm wide and 6 - 10 cm long, the base of leaves is oblique and the leaves are glabrous yellowish green. Flowers are bisexual, zygomorphic petal and white color in short auxiliary clusters. The bract is small. The calyx is divided into 5 deeply acute parted, light green and 5 - 6 mm long. The corolla tube is bilabiate, upper lip erect, bifid and lower lips 3 lobed. The corolla has brownish purples spots at the throat of the tube. There are 4 stamens with didynamous. The ovary is superior with 2-loculed and ovule free placentation. The fruit is a capsule (Panichayupakaranant *et al*., 2006).

2.2 Ecology and propagation of *R. nasutus*

R. nasutus is locally known and widely distributed in tropical countries. It is scattered along the edges of evergreen forests. *R. nasutus* plants are usually grown as ornamentals and require sandy and well-drained soil. They can be propagated by seeds or cutting.

Figure 2.1 *Rhinacanthus nasutus* (L.) Kurz

2.3 Distribution of rhinacanthins in *R. nasutus* and effect of harvesting period

Determination of total rhinacanthin content in the leaves, stems, and roots of *R. nasutus*, which were collected at a different period of times, has demonstrated that rhinacanthins markedly accumulated in the roots and leaves, but less so in the stems of the plant (Table 2.1). Regarding the effect of harvesting period, it was found that the leaves and roots harvested in July yielded higher amounts of rhinacanthins. In July, *R. nasutus* is not yet in bloom. Thus, *R. nasutus* leaves and roots should be harvested before blossom. Although the leaves and roots that were harvested in other periods gave a lower content of rhinacanthins, they still passed the lower limit of the total rhinacanthins (Panichayupakaranant *et al*., 2006).

Table 2.1 Rhinacanthin content in leaves, stems and roots of *R. nasutus* harvested in different times

Period of harvesting	**Rhinacanthin content (%w/w)**		
	Leaves	**Stems**	**Roots**
April 2003	3.6 ± 0.1	2.1 ± 0.0	4.3 ± 0.3
July 2003	5.6 ± 0.0	1.0 ± 0.0	5.7 ± 0.1
October 2003	4.4 ± 0.1	0.6 ± 0.0	4.7 ± 0.0
January 2004	3.3 ± 0.0	0.8 ± 0.0	4.2 ± 0.0

2.4 Ethnomedical uses of *R. nasutus*

R. nasutus has long been used in Thai traditional medicine for skin diseases such as pruritis, tinea versicolor, and ringworm. The traditional recipes for treatment of ringworm are as follows (Farnsworth and Bunyapraphatsara, 1992).

- A tincture is prepared by soaking fresh leaves and roots in alcohol. Then it is applied over the infected area.

- The roots (6 -7 roots) are pounded with match tips and vaseline then it is applied over infect area.

- The roots are pounded with lemon and tamarind juices then the mixture is applied over the infected area.

Pounded roots mix with vinegar or alcohol was applied on herpetic-like eruptions. For the same purpose, the leaves are applied with benzoin and sulfur in Malaysia. In Indonesia, the flowers and young leaves are rubbed with vinegar and lime to the skin (Wiart *et al*., 2000).

2.5 Chemical constituents of *R. nasutus*

The groups of secondary metabolites commonly found in *R. nasutus* are naphthoquinones. List of the compounds found in *R. nasutus* is shown in Table 2.2. Structures of some compounds are given in Figure 2.2, 2.3, 2.4 and 2.5, respectively.

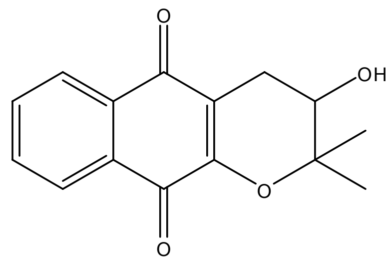
Table 2.2 Chemical constituents of *Rhinacanthus nasutus*

Chemicals	Plant parts	References
1. Naphthoquinones		
rhinacanthin-A	Roots	Wu *et al*., 1988; Wu *et al*., 1998a; Wu *et al*., 1998b; Singh *et al*., 1992
rhinacanthin-B	Roots	Wu *et al*., 1988; Wu *et al*., 1998a; Wu *et al*., 1998b
rhinacanthin-C	Whole plants	Sendl *et al*., 1996; Wu *et al*., 1998a; Wu *et al*., 1998b
rhinacanthin-D	Whole plants	Sendl *et al*., 1996; Wu *et al*., 1998a; Wu *et al*., 1998b
rhinacanthin-G	Roots	Wu *et al*., 1998a; Wu *et al*., 1998b
rhinacanthin-H	Roots	Wu *et al*., 1998a; Wu *et al*., 1998b
rhinacanthin-I	Leaves and roots	Wu *et al*., 1998a; Wu *et al*., 1998b

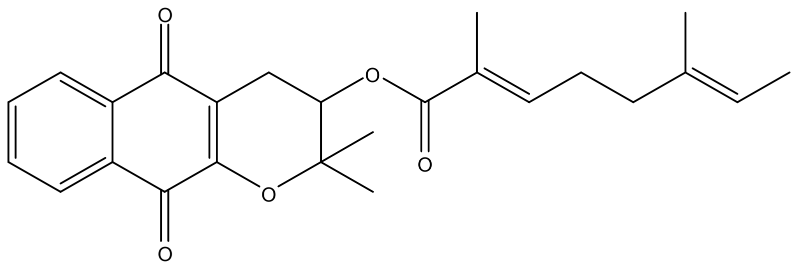
Chemicals	Plant parts	References
rhinacanthin-J	Leaves and roots	Wu *et al*., 1998a;
		Wu *et al*., 1998b
rhinacanthin-K	Roots	Wu *et al*., 1998a;
		Wu *et al*., 1998b
rhinacanthin-L	Roots	Wu *et al*., 1998a;
		Wu *et al*., 1998b
rhinacanthin-M	Roots	Wu *et al*., 1998a;
		Wu *et al*., 1998b
rhinacanthin-N	Leaves and roots	Wu *et al*., 1998a;
		Wu *et al*., 1998b
rhinacanthin-O	Roots	Wu *et al*., 1998a;
		Wu *et al*., 1998b
rhinacanthin-P	Roots	Wu *et al*., 1998a;
		Wu *et al*., 1998b
rhinacanthin-Q	Roots	Wu *et al*., 1998b
rhinacanthone	Leaves and stems	Kodama *et al*., 1993; Kuwahara *et al*., 1995
dehydro-α-lapachone	Roots	Wu *et al*., 1998a;
		Wu *et al*., 1998b
2. Lignans		
rhinacanthin-E	Aerial parts	Kernan *et al*., 1997
rhinacanthin-F	Aerial parts	Kernan *et al*., 1997

Chemicals	Plant parts	References
3. Benzenoids		
p-Hydroxy-benzaldehyde	Roots	Wu *et al*., 1998b
vanillic acid	Leaves and stems	Wu *et al*., 1995
syringic acid	Leaves and stems	Wu *et al*., 1995
2-methoxy-4 - propionylphenol	Leaves and stems	Wu *et al*., 1995
methyl valinate	Roots	Wu *et al*., 1998b
syringaldehyde	Roots	Wu *et al*., 1998b
4. Anthraquinone		
2-methyl anthraquinone	Leaves and stems	Wu *et al*., 1995
5. Triterpenoids		
β-amyrin	Roots	Wu *et al*., 1995
glutinol	Roots	Wu *et al*., 1995
lupeol	Roots	Wu *et al*., 1988; Wu *et al*., 1995; Wu *et al*., 1998b
6. Flavonoids		
wogonin	Roots	Wu *et al*., 1998b
7. Sterols		
stigmasterol	Roots	Wu *et al*., 1988
β-sitosterol	Roots	Wu *et al*., 1988
8. Chlorophyll		
methylpheophorbide-A	Leaves and stems	Wu *et al*., 1995

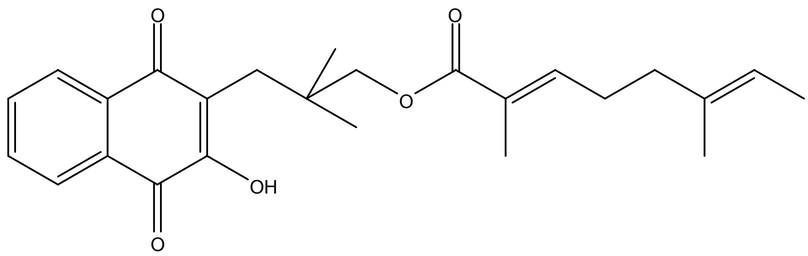
Chemicals	Plant parts	References
9. Coumarins		
(+)-praeruptorin	Roots	Wu *et al.*, 1998b
umbelliferone	Leaves and stems	Wu *et al.*, 1995
10. Amide		
allantoin	Roots	Wu *et al.*, 1998b
11. Carbohydrate		
methyl-α-D-galactopyranoside	Leaves and stems	Wu *et al.*, 1995
12. Quinol		
4-acetonyl-3,5-dimethoxy-p-quinol	Leaves and stems	Wu *et al.*, 1995
13. Benzoquinone		
2,6-dimethoxy benzoquinone	Leaves and stems	Wu *et al.*, 1995
14. Glycosides		
sitosterol-β-D-glucopyranoside	Leaves and stems	Wu *et al.*, 1995
stigmasterol-β-D-glucopyranoside	Leaves and stems	Wu *et al.*, 1995
3,4-dimethylphenol-β-D-glucopyranoside	Leaves and stems	Wu *et al.*, 1995
3,4,5-trimethylphenol-β-D-glucopyranoside	Leaves and stems	Wu *et al.*, 1995



rhinacanthin-A

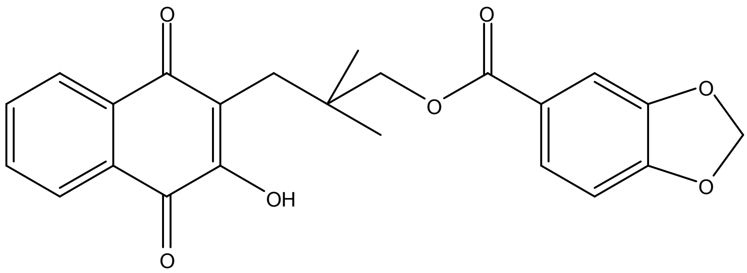


rhinacanthin-B

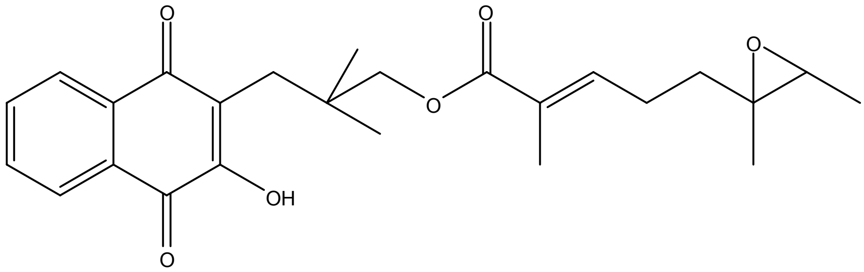


rhinacanthin-C

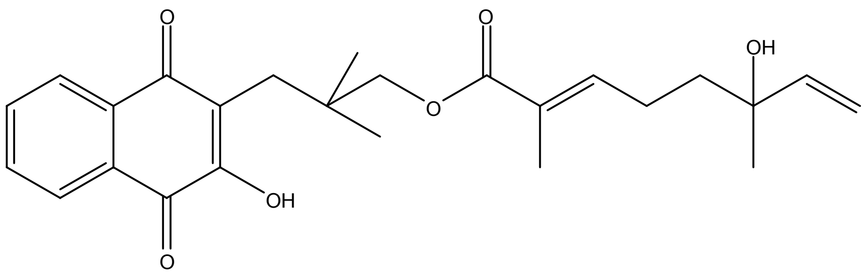
Figure 2.2 Structure of rhinacanthins



rhinacanthin-D

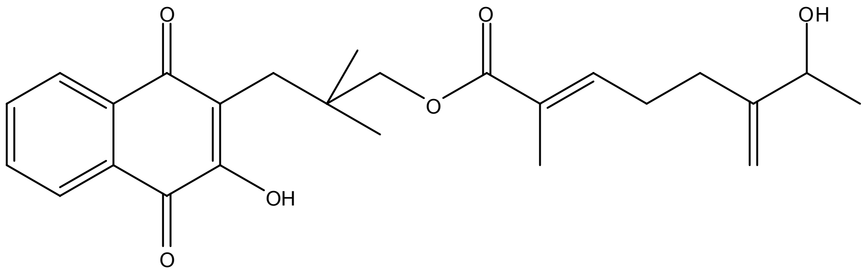


rhinacanthin-G

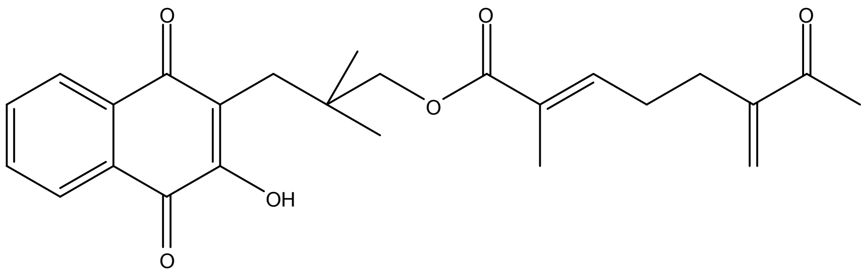


rhinacanthin-H

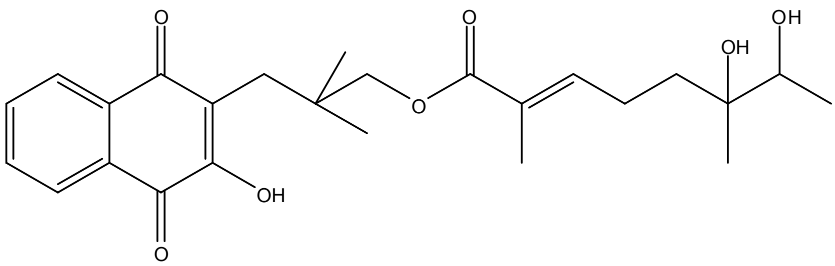
Figure 2.2 Structure of rhinacanthins (continued)



rhinacanthin-I

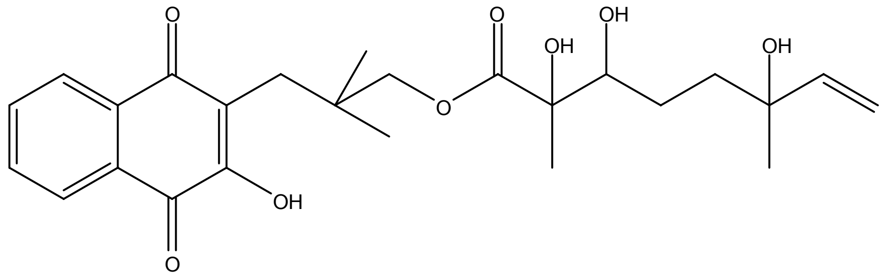


rhinacanthin-J

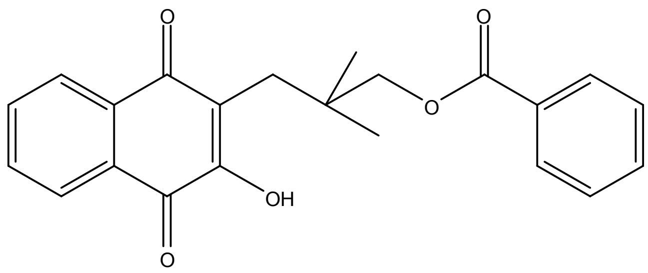


rhinacanthin-K

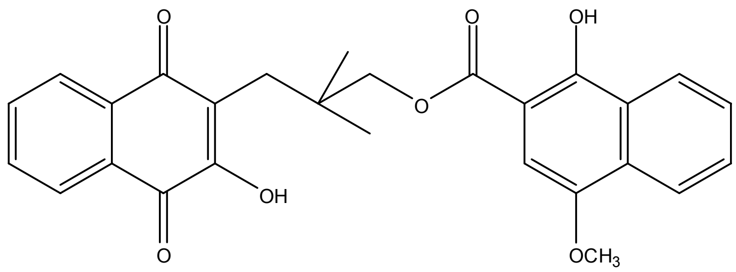
Figure 2.2 Structure of rhinacanthins (continued)



rhinacanthin-L

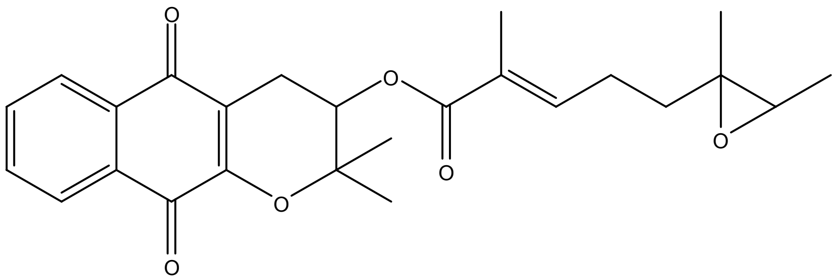


rhinacanthin-M

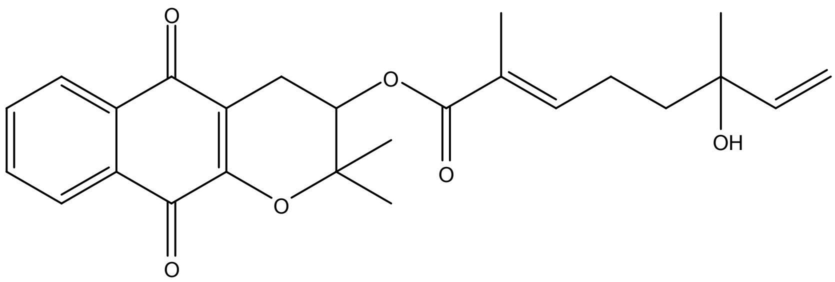


rhinacanthin-N

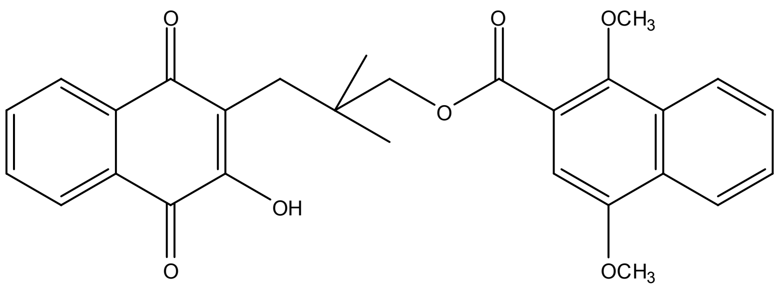
Figure 2.2 Structure of rhinacanthins (continued)



rhinacanthin-O

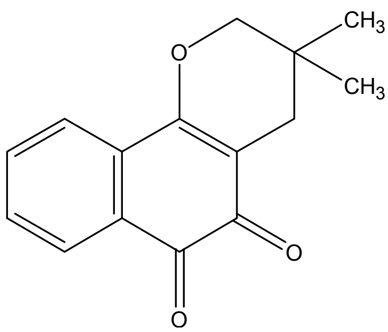


rhinacanthin-P

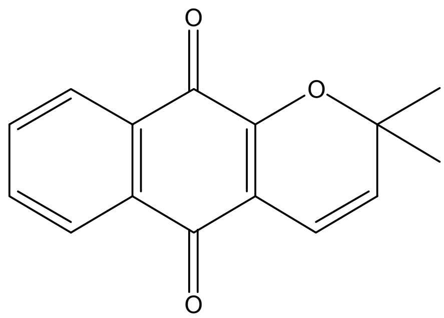


rhinacanthin-Q

Figure 2.2 Structure of rhinacanthins (continued)

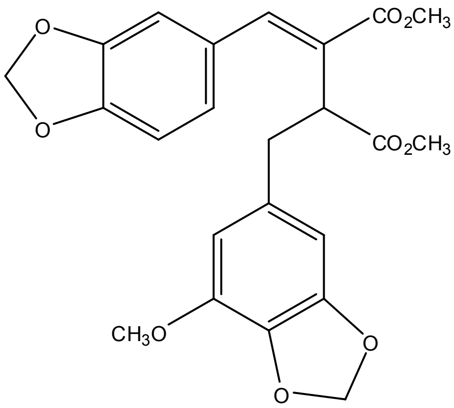


rhinacanthone

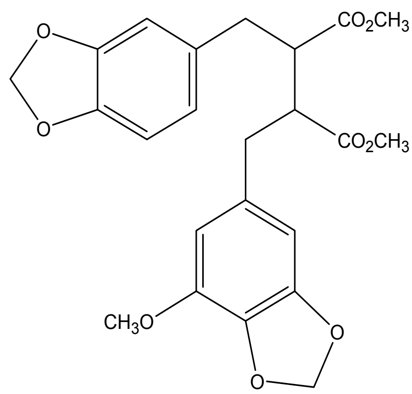


dehydro-α-lapachone

Figure 2.3 Structure of rhinacanthone and dehydro-α-lapachone

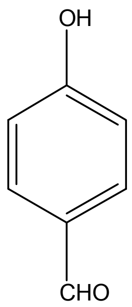


rhinacanthin-E: Δ7E

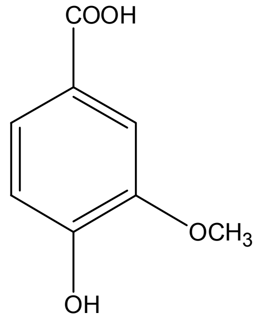


rhinacanthin-F: Δ7Z

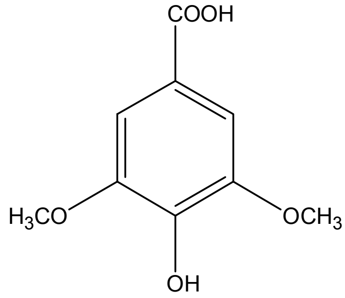
Figure 2.4 Structure of lignans



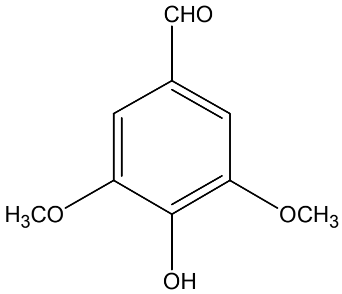
p-Hydroxy benzaldehyde



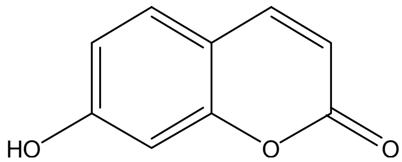
vanillic acid



syringic acid



syringaldehyde



umbelliferone

Figure 2.5 Structure of other phenolic compounds

2.6 Biological activity of *R. nasutus* and rhinacanthins

R. nasutus has long been used as a folk medicine in Thailand. The properties mostly acknowledged for a long time are treated of tinea vesicolor and ringworm (Farnsworth and Bunyapraphatsara, 1992). Moreover, it has been reported that the rhinacanthins shown interesting pharmacological activities as follow:

2.6.1 Antifungal activity

It has been reported that rhinacanthin-C, -D and -N isolated from *R. nasutus* leaves exhibited antifungal activity *Microsporum gypseum*, *Trichophyton rubrum* and *T. mentagrophytes* that cause tinea in human (Kongchai and Panichayupakaranant, 2002). The MIC values were showed in Table 2.3. Furthermore, there was a report on antifungal activity of rhinacanthin-C, -D and -N in *R. nasutus* leaves extract against *Candida albicans* and the MIC values were 512, 64 and 64 μg/ml, respectively (Panichayupakaranant *et al*., 2000).

Table 2.3 Antifungal activity of rhinacanthins isolated from the leaves of *R. nasutus*

Compounds	**MIC (μg/ml)**		
	T. rubrum	***T. mentagrophytes***	***M. gypseum***
Rhinacanthin-C	31.2	31.1	125
Rhinacanthin-D	62.5	62.5	250
Rhinacanthin-N	125	125	250

2.6.2 Antiviral activity

Sendl and his group (Sendl *et al*., 1996) had studied on antiviral activity of rhinacanthin-C and rhinacanthin-D against cytomegalovirus in mice (mCMV) and human (hCMV), influenza virus type A, herpes simplex virus type 2 and respiratory syncytial virus. The result showed a good activity of rhinacanthin-C and rhinacanthin-D against hCMV with the IC_{50} values of 0.02 and 0.22 μg/ml, respectively.

2.6.3 Cytotoxic activity

Wu and his group (Wu *et al*., 1988b) had studied on cytotoxic activity of the naphthoquinones isolated from the roots of *R. nasutus* including rhinacanthin-A, -B, -C, -G, -H, -I, -K, -L, -M, -N and -Q against murine leukemia (P-388), human lung carcinoma (A-549), human colon adenocarcinomar (HT-29), and leukemia (HL-60) cells with the ED_{50} values as shown in Table 2.4.

Table 2.4 Cytotoxic activity of rhinacanthins isolated from the roots of *R. nasutus*

Compounds	Cell lines ED_{50} (µg/ml)				
	KB	P-388	A-549	Ht-29	HL-60
Rhinacanthin-A	6.75	0.72	3.06	2.17	1.16
Rhinacanthin-B	8.01	0.35	6.50	3.01	2.57
Rhinacanthin-C	6.26	0.26	0.35	0.68	0.68
Rhinacanthin-D	25.0	3.79	8.26	8.89	11.8
Rhinacanthin-G	4.45	0.14	0.75	0.57	1.14
Rhinacanthin-H	23.8	6.43	9.97	11.5	8.87
Rhinacanthin-I	13.2	4.88	7.18	6.30	5.12
Rhinacanthin-K	17.3	3.17	16.4	7.75	6.81
Rhinacanthin-M	19.2	3.95	8.90	10.1	19.9
Rhinacanthin-N	4.80	0.71	1.97	2.67	1.38
Rhinacanthin-Q	>50	0.61	3.61	7.60	8.90

Panichayupakaranant and his group (Panichayupakaranant *et al*., 2003) had studied on anticancer activity of rhinacanthin-C, -D and -N isolated from *R. nasutus* leaves against human cervical carcinoma (HeLa) and human Caucasian breast adenocarcinoma (MCF-7). Rhinacanthin-C shown the ED_{50} values of 0.85 and 1.02 µg/ml, rhinacanthin-D shown the ED_{50} values of 14.54 and 3.34 µg/ml and rhinacanthin-N show the ED_{50} values of 1.59 and 2.78 µg/ml against HeLa and MCF-7 cell lines, respectively. Gotoh and his group (Gotoh *et al*., 2004) had studied on in vitro antiproliferative activity of the leaf extract and root extracts of *R. nasutus* and rhinacanthin-C against HeLa, MDR 1-overexpressing subline of human cervical carcinoma (Hvr-100-6), human prostatic cancer cell (PC-3), and human bladder (T24) carcinoma. The result showed an antiproliferlative activity of all treated compounds with the IC_{50} values as shown in Table 2.5.

Table 2.5 *In vitro* antiproliferlative activities of the *R. nasutus* extracts and rhinacanthin-C in tested cell lines

Compounds	IC_{50}			
	HeLa	Hvr-100-6	PC-3	T24
Root extract (µg/ml)	1239	977	567	373
Leaf extract (µg/ml)	1499	1582	359	616
Rhinacanthin-C (µM)	26.2	11.2	1.92	0.66

In addition, Siripong and his group (Siripong *et al*., 2006) had studied on antiproliferative activity of rhinacanthin-C, -N and -Q isolated from the roots of *R. nasutus*. It was found that rhinacanthin-C, -N and -Q were capable of inhibiting cell proliferation apoptosis of human cervical carcinoma cell (HeLS3) in dose and time dependent manners.

2.6.4. Antiplatelet activity

The antiplatelet aggregation of naphthoquinones, which isolated from the roots of *R. nasutus* including rhinacanthin-A, -B, -C, -G, -H, -I, -K, -M, and -Q, have been reported. These compounds demonstrated 36 - 100% inhibition of rabbit platelet aggregation induced by arachidonic acid (100 mM). Rhinacanthin-A, -B, and -C (10 µg/ml) showed 72-100% inhibition of the rabbit platelet aggregation induced by collagen, while rhinacanthin-B (2 ng/ml) inhibited platelet aggregation induced by platelet activation factor (Wu *et al*., 1998b).

2.7 Naphthoquinone production by plant cell and tissue cultures

Plant cell cultures represent a potential source of valuable secondary metabolites which can be used as food additives, nutraceuticals, and pharmaceuticals (Zhong, 2001). The problems related to obtaining of secondary metabolites from plants include environmental factors, political and labor instabilities in the producing countries, uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, and losses in storage and handling. In many cases, the chemical synthesis of these compounds is either extremely difficult or economically infeasible (Namdeo, 2007). The production of useful and valuable secondary metabolites from cell cultures is an attractive proposal. Cell culture technology was developed as a possible tool to both study and produce plant secondary metabolites. The evolving importance of the secondary metabolites has resulted in a high level of interest in the possibility of altering their production through improving cultivation technology (Chong, 2001).

Many techniques have been successfully used for improving secondary metabolite production by plant tissue cultures such as medium manipulation, elicitation, cell immobilization, cell line selection, precursor addition, hairy root cultures and genetic transformation (Dornenburg and Knorr, 1995; Abdullah *et al*., 2005). In this studied R. nasutus tissue cultures were established and induced of rhinacanthin formation by using medium manipulation and elicitation techniques.

Manipulation of the culture environment must be effective in increasing the product accumulation. The expression of many secondary metabolite pathways is easily altered by external factors such as nutrient levels, light and plant growth regulators. Many of the constituents of plant cell culture media are important determinants of growth and accumulation of secondary metabolites (Stafford *et al*., 1986; Misawa, 1985). Plant growth regulator concentration is often a crucial factor in secondary product accumulation (Deus and Zenk, 1982). The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alters dramatically both growth and product formation in cultured plant cells (Mantell and Smith, 1984). For example, 2,4-D has been shown to inhibit the production of secondary metabolites in a large number of cases. In such cases, elimination of 2,4-D or replacement of 2,4-D by NAA or IAA has been shown to enhance the production of anthocyanins in suspension cultures of *Populus deltoides* and *Daucus carota*, betacyanins in suspension cultures of *Portulaca*, nicotine in suspension cultures of *Nicotiana tabacum* and shikonin in suspensions of *Lithospermum erythrorhizon* (Sahai and Shuler, 1984; Tabata, 1985; Seitz and Hinderer, 1988; Rajendran *et al*., 1992). However, stimulation by 2,4-D has been observed in carotenoid biosynthesis in suspensions of *Daucus carota* (Mok *et al*., 1976) and in anthocyanin production in callus cultures of *Oxalis linearis* (Meyer and Staden, 1995). As cytokinins have different effects depending on the type of metabolite and species concerned. For example, kinetin stimulated the production of anthocyanins in *Haplopappus gracilus* but inhibited the formation of anthocyanins in *Populus deltoides* cell cultures (Mok *et al*., 1976; Seitz and Hinderer, 1988). Several types of products related with naphthoquinones have been successfully elevated by elicitation as shown in Table 2.6.

Table 2.6 Example of naphthoquinone production by medium manipulation

Plant	Naphthoquinone	Culture type	References
Drosera binata	Plumbagin	Suspension	Hook, 2001
Drosera capensis	7-methyljuglone	Suspension	Hook, 2001
Dionaea muscipula	Plumbagin	Suspension	Hook, 2001
Drosera rotundifolia	7-methyljuglone	Suspension	Hook, 2001
Impatiens balsamina	Lawsone	Suspension	Panichayupakaranant, 2001
Impatiens balsamina	Lawsone and methyl lawsone	Suspension	Panichayupakaranant and De-Eknamkul, 1992
Lawsoniainermis	Lawsone	Root culture	Bakkali *et al*., 1997
Lithospermum erythrorhizon	Shikonin	Callus culture	Mizukami *et al*., 1997
Lithospermum erythrorhizon	Shikonin	Suspension	Fujita *et al*., 1981
Plumbago rosea	Plumbagin	Root culture	Panichayupakaranant and Tewtrakul, 2002

Elicitation is one of the most effective biotechnological approaches to induce or enhance biosynthesis of metabolites by biotic or abiotic molecules, that so-called "elicitors" (Radman *et al*., 2003). Elicitors are compounds which are able to trigger defense mechanisms such as hypersensitive response, production of reactive oxygen species and activation of defense-related as well as phytoalexin synthesis (Smith, 1996; Ebel and Mithofer, 1998). Examples of biotic elicitors are bacteria and fungal cell wall; abiotic elicitors are UV light, temperature, heavy metals, etc (Singh, 1999; Chong *et al*., 2005). In general, modes of action for biotic are divided into four types of interaction (Becker and Sauerwein, 1990) as follow:

1. Elicitors directly released by the microorganism and recognized by the plant cell.
2. Elicitors formed by action of microorganism on plant cell wall.
3. Elicitors formed by action of plant enzymes on microbial cell walls.
4. Elicitor compounds, endogenous and constitutive in nature, formed or released by the plant cell in response to various stimuli.

The effectiveness of elicitation is depended on a complex interaction between the elicitor and the plant cell. However, there are some of the main factors affected this interaction and thereby the elicitation response such as elicitor specificity, elicitor concentration, treatment interval and culture conditions (growth stage, medium composition and light), affected the secondary metabolite production in different plant species. Several types of products related with naphthoquinones have been successfully elevated by elicitation as shown in Table 2.7.

Table 2.7 Example of naphthoquinone production by elicitation techniques

Plant	Elicitor	Naphthoquinone	Culture type	References
Arnebia euchroma	Fungi	Shikonin	Suspension	Fu and Lu, 1999
Drosera capensis	Salicylic acid and jasmonic acid	7-methyljuglone	Root culture	Ziaratnia *et al*., 2009
Lithospermum erythrorhizon	Methyl jasmonate	Shikonin	Suspension	Yazaki *et al*., 1997
Lithospermum erythrorhizon	Fungi	Shikonin	Suspension	Kim *et al*., 1990
Plumbago rosea	Chitosan, fungi, bacteria and yeast extract	Plumbagin	Suspension	Komaraiah *et al*., 2002

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

R. nasutus leaves were collected from the botanical garden, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla.

3.1.2 Chemicals

Standard rhinacanthin-D, -C, and -N were previously purified by Assoc. Prof. Dr. Pharkphoom Panichayupakaranant (Panichayupakaranant, *et al* 2003). *Trichophyton rubrum* and *Candida albicans* were obtained from Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Chemicals used in this study are shown in Table 3.1.

Table 3.1 List of chemicals

Chemical	Company, Country
1-Naphthylacetic acid (NAA)	Fluka, Switzerland
2,4-Dichlorophenoxyacetic acid (2,4-D)	Fluka, Switzerland
6-Benzylaminopurine (BA)	Fluka, Switzerland
3-Indolebutylic acid (IBA)	Fluka, Switzerland
3-Indoleacetic acid (IAA)	Fluka, Switzerland
Ammonium nitrate (NH_4NO_3)	Nuplex Industries Pty Ltd, Australia
Ammonium sulfate ($(NH_4)_2$. SO_4)	Merck, Germany
Boric acid (H_3BO_3)	Fisher Scientific, England
Calcium chloride ($CaCl_2$. $2H_2O$)	Merck, Germany
Calcium nitrate ($Ca(NO_3)_2$. $4H_2O$	Analar, England
Chitosan	Wako Pure Chemical Industries,Ltd, China
Clorox®	Clorox, Malaysia
Cobalt chloride ($CoCl_2$. $6H_2O$)	Fluka, Switzerland
Cupric sulfate ($CuSO_4$. $5H_2O$)	Merck, Germany
Dimethyl sulfoxide, AR grade	Riedel-de Haen, Germany
Ethanol (95%v/v)	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Ethyl acetate, commercial grade	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Glacial acetic acid	Baker, USA

Table 3.1 List of chemicals (Continued)

Chemical	Company, Country
Glycine	Sigma, Germany
Ferrous sulfate ($FeSO_4$. $7H_2O$)	Fisher Scientific, England
Kinetin (Kn)	Fluka, Switzerland
Manganese sulfate ($MnSO_4$. H_2O)	Fluka, Switzerland
Magnesium sulfate ($MgSO_4$. $7H_2O$)	APS Ajax Finechem, Australia
Methanol HPLC grade	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Methyl jasmonate	Sigma Aldrich, Germany
Myo - inositol	VWR International Ltd, England
Nicotinic acid	Fluka, Switzerland
Plant agar	Duchefa Biochemic, The Netherlands
Potassium Iodine (KI)	Merck, Germany
Potassium nitrate (KNO_3)	VWR International Ltd, England
Potassium phosphate (KH_2. PO_4)	Sigma, Germany
Potassium sulfate (K_2SO_4)	Baker, USA
Pyridoxine hydrochloride	Fluka, Switzerland
Sodium molybdate (Na_2MoO_4. $2H_2O$)	Merck, Germany
Sodium phosphate (NaH_2PO_4. H_2O)	Sigma, Germany
Sucrose	MITRPOL, Thailand
Sodium ethylenediaminetetraacetic acid (Na_2EDTA)	Sigma, Germany

Table 3.1 List of chemicals (Continued)

Chemical	Company, Country
Thiamine hydrochloride	Sigma, Germany
Thidiazuron (TDZ)	Merck, Germany
Zinc Sulfate ($ZnSO_4$. $7H_2O$)	Fluka, Switzerland

3.1.3 Instrumentations

Table 3.2 List of instrumentations

Instrumentation	Company, Country
Autoclave machine, Model HA-3D	Hirayama, Japan
Centrifuge, Kubota 5922	Kubota corporation, Japan
Hot air oven, Memmert	Schwubuch, Germay
Hot plate and stirrer, CORNING	Fisher Scientific, USA
HPLC, Agilent 1100 series	Palo Alto, U.S.A
HPLC column, TSK-GEL ODS-80Tm	Tosho Bioscience, Japan
Laminar air flow cabinet, HT-122	Holten, Denmark
Micropipette, ACURA 825	Orion Research, Switzerland
Hot air oven, DIN 12880-KI	Memmert, Germany
pH meter, Model 710 A	Thermo Electric company, USA
Rotary evaporator, N 1000	EYELA, Japan
Shaker, Innova 2300	Illinois, USA
Sonicator, S 100H	Crest Ultrasonic Corporation, USA

3.2 Methods

3.2.1 Preparation of *R. nasutus* leaf explant

The young leaves of *R. nasutus* were washed with running tap water for 2 hours and rinsed 3 times with distilled water. The leaves were then dipped into 70% v/v ethanol for 10 seconds and subsequently soaked into 20% v/v Clorox® solution for 15 minutes. After that, the sterile leaves were rinsed 3 times with sterile distilled water and cut with a sharp scalpel. The explants were then transferred to solid media.

3.2.2 Preparation of media

The Formulations of the cultured media, Gamborg (B5), Murashige & Skoog (MS) and Woody Plant Medium (WPM) are shown in Table 3.3. Stock solutions of B5, MS and WPM are shown in Table 3.4, 3.5 and 3.6, respectively. The stock solutions of various plant growth regulators are shown in Table 3.7.

Table 3.3 Nutrient composition of MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968) and WPM media (Owen and Miller, 1992)

Constituent	**Concentration (mg/l)**		
	MS	**B5**	**WPM**
Macronutrients:			
KNO_3	1900	2528	-
$MgSO_4.7H_2O$	370	250	370
$CaCl_2.2H_2O$	440	150	96
$(NH_4)_2.SO_4$	-	134	-
NH_4NO_3	1650	-	400
KH_2PO_4	170	-	170
K_2SO_4	-	-	990
$Ca(NO_3)_2.4H_2O$	-	-	556
Micronutrients:			
$MnSO_4$. $4H_2O$	22.3	10.0	22.3
$FeSO_4$. $7H_2O$	27.8	27.8	27.8
NaH_2PO_4 H_2O	-	150	-
H_3BO_3	6.2	3.0	6.2
$ZnSO_4.7H_2O$	8.6	2.0	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
KI	0.83	0.75	-
$CuSO_4.5H_2O$	0.025	0.025	0.25
$CoCl_2.6H_2O$	0.025	0.025	-
Na_2EDTA	37.2	37.2	7.2
Sucrose (g)	30	20	20
Vitamins:			
Thiamine HCl	0.1	10	1
Pyridoxine HCl	0.5	1	0.5
Nicotinic acid	0.5	1	0.5
Glycine	2.0	-	2.0
Myo-Inositol	100	100	100

Table 3.4 Stock solutions of B5 medium

B5		Remarks
Stock 1 (Macronutrients)	g/1000 ml	Store in refrigerator
KNO_3	50.56	
NaH_2PO_4 H_2O	3.0	
$(NH_4)_2.SO_4$	2.68	
$MgSO_4.7H_2O$	5.0	
Stock 2 (Micronutrients)	g/100 ml	Store in refrigerator
$MnSO_4$. $4H_2O$	1	
H_3BO_3	0.3	
$ZnSO_4.7H_2O$	0.2	
$NaMoO_4.2H_2O$	0.025	
$CuSO_4.5H_2O$	0.0025	
$CoCl_2.6H_2O$	0.0025	
Stock 3 (Ca stock)	g/100 ml	Store in refrigerator
$CaCl_2.2H_2O$	15	
Stock 4 (KI stock)	g/100 ml	Store in amber in bottle refrigerator
KI	0.075	
Stock 5 (Vitamins)	g/100 ml	Store in freezer (10 ml fraction)
Thiamine HCl	1.0	
Pyridoxine HCl	0.10	
Nicotinic acid	0.10	
Myo-Inositol	10	
Stock 6 (Fe-EDTA stock)	g/500 ml	Store in refrigerator
Na_2.EDTA	3.73	
$FeSO_4.7H_2O$	2.78	

Table 3.5 Stock solutions of MS medium

MS		Remarks
Stock 1 (Macronutrients)	g/1000 ml	Store in refrigerator
KNO_3	38.0	
NH_4NO_3	33.0	
$MgSO_4.7H_2O$	7.4	
KH_2PO_4	3.4	
Stock 2 (Micronutrients)	g/100 ml	Store in refrigerator
$MnSO_4.H_2O$	1.69	
H_3BO_3	0.62	
$ZnSO_4.7H_2O$	0.86	
$Na_2MoO_4.2H_2O$	0.0025	
$CuSO_4.5H_2O$	0.0025	
$CoCl_2.6H_2O$	0.0025	
Stock 3 (Ca stock)	g/100 ml	Store in refrigerator
$CaCl_2.2H_2O$	44	
Stock 4 (KI stock)	g/100 ml	Store in amber in bottle refrigerator
KI	0.083	
Stock 5 (Vitamins)	g/100 ml	Store in freezer (10 ml fraction)
Nicotinic acid	0.5	
Thiamine HCl	0.1	
Pyridoxine HCl	0.5	
Glycine	0.2	
Myo-Inositol	10	
Stock 6 (Fe-EDTA stock)	g/500 ml	Store in refrigerator
$Na_2.EDTA$	3.73	
$FeSO_4.7H_2O$	2.78	

Table 3.6 Stock solutions of WPM medium

WPM		Remarks
Stock 1 (Macronutrients)	g/1000 ml	Store in refrigerator
K_2SO_4	19.8	
NH_4NO_3	8.0	
$MgSO_4.7H_2O$	7.4	
KH_2PO_4	3.4	
Stock 2 (Micronutrients)	g/100 ml	Store in refrigerator
$MnSO_4.H_2O$	2.23	
H_3BO_3	0.62	
$ZnSO_4.7H_2O$	0.86	
$CuSO_4.5H_2O$	0.025	
$Na_2MoO_4.2H_2O$	0.025	
Stock 3 (Ca stock)	g/500 ml	Store in amber in bottle refrigerator
$CaCl_2.2H_2O$	2.4	
$Ca(NO_3)_2.4H_2O$	13.9	
Stock 4 (Vitamins)	g/100 ml	Store in freezer (10 ml fraction)
Pyridoxine HCl	0.005	
Nicotinic acid	0.005	
Thiamine HCl	0.01	
Glycine	0.002	
Myo-Inositol	1	
Stock 5 (Fe-EDTA stock)	g/500 ml	Store in refrigerator
$Na_2.EDTA$	0.746	
$FeSO_4.7H_2O$	2.78	

Table 3.7 Stock solutions of plant growth regulators

Plant growth regulators		Remarks
BA stock solution	mg/100 ml	Dissolve BA in a small 0.5 N HCl and
BA	10	dilute to 100 ml with dilled water. Store in refrigerator.
Kn stock solution	mg/100 ml	Dissolve Kn in a small 0.5 N HCl and
Kn	10	dilute to 100 ml with dilled water. Store in refrigerator.
TDZ stock solution	mg/1 ml	Dissolve TDZ in 1 ml DMSO and store
TDZ	10	in refrigerator.
2,4-D stock solution	mg/100 ml	Dissolve 2,4-D in 5 ml ethanol and
2,4-D	10	dilute to 100 ml with dilled water. Store in refrigerator.
NAA stock solution	mg/100 ml	Dissolve NAA in 5 ml ethanol and
NAA	10	dilute to 100 ml with dilled water. Store in refrigerator.
IBA stock solution	mg/100 ml	Dissolve IBA in 5 ml ethanol and dilute
IBA	10	to 100 ml with dilled water. Store in refrigerator.
IAA stock solution	mg/100 ml	Dissolve IAA in 5 ml ethanol and dilute
IAA	10	to 100 ml with dilled water. Store in refrigerator.

B5, MS and WPM were prepared from their stock solutions. The stock solutions of each medium as shown in Table 3.8 - Table 3.10 were added into distilled water (80% of the final required volume) followed by stirring. Sucrose and plant growth regulators stock solutions (as needed) were then added and stirring continued until complete dissolution. The cultured medium was then adjusted to the final volume (1 l) with distilled water. The pH of B5, MS and WPM were adjusted to 5.5, 5.8 and 5.7, respectively. In the case of solid and semi-solid medium, agar (0.8 and 0.4 % w/v, respectively) was added and heated gently with continuous stirring until complete dissolution. The culture media was subjected to sterilization by autoclaving at 121 ºC, 15 lb/in^2, for 15 minutes.

Table 3.8 Preparation of B5 medium

B5		
Distilled water	1000	ml
Stock 1	50	ml
Stock 2	1.0	ml
Stock 3	1.0	ml
Stock 4	1.0	ml
Stock 5	1.0	ml
Stock 6	5.0	ml
Sucrose	20	g
Agar (solid medium)	8	g
Auxin (100 mg/l) as needed		
Cytokinin (100 mg/l) as needed		
Final pH adjust to pH 5.5		

Table 3.9 Preparation of MS medium

MS		
Distilled water	1000	ml
Stock 1	50	ml
Stock 2	1.0	ml
Stock 3	1.0	ml
Stock 4	1.0	ml
Stock 5	1.0	ml
Stock 6	5.0	ml
Sucrose	30	g
Agar (solid medium)	8	g
Agar (semisolid medium)	4	g
Auxin (100 mg/l) as needed		
Cytokinin (100 mg/l) as needed		
Final pH adjust to pH 5.8		

Table 3.10 Preparation of WPM medium

WPM		
Distilled water	1000	ml
Stock 1	50	ml
Stock 2	20	ml
Stock 3	1.0	ml
Stock 4	10	ml
Stock 5	5.0	ml
Sucrose	20	g
Agar (solid medium)	8	g
Auxin (100 mg/l) as needed		
Cytokinin (100 mg/l) as needed		
Final pH adjust to pH 5.8		

3.2.3 Establishment of *R. nasutus* callus cultures

The young leaf explants of *R. nasutus* were cultured on solid B5 medium supplemented with a combination of 2.0 mg/l BA and various concentration of IBA (0.1, 0.5, 1.0 and 2.0 mg/l). The cultures were incubated under 16 h light (light intensity is 1,000 lux), 8 hours dark. Subculture of the callus cultures was performed at 6 weeks old. The callus formation and morphological appearance were observed. The callus formation was graded in 5 levels: 0 = none, 1 = least, 2 = little, 3 = medium, 4 = much 5 = very much.

3.2.4 Establishment of *R. nasutus* cell suspension cultures

The cell suspension cultures of *R. nasutus* were initiated by transferring the callus into liquid B5 medium containing 2.0 mg/l BA and 0.5 mg/l IBA. The cell suspension cultures were incubated in a rotary shaken erlenmeyer flask (50 ml medium in 250 ml-flask; 150 rpm) at 25^{o}C under dark conditions. Maintenance of the cultures was carried out by periodic subculture into the same liquid B5 medium at 4-week intervals.

3.2.5 Medium manipulation

The effect of auxin and cytokinin on rhinacanthin production of *R. nasutus* cell suspension cultures was determined. Four types of auxin (NAA, 2,4-D, IBA and IAA) and two types of cytokinin (BA and TDZ) at the concentration of 2.0 mg/l were examined. Eight combinations of auxin and cytokinin in B5 were obtained as shown in Table 3.11. The cell suspension cultures in each medium were harvested when they are 4 weeks old and subjected to determination of the rhinacanthin content by HPLC.

Table 3.11 The combination of cytokinins and auxins in B5 medium for induction of rhinacanthin production experiment

Cytokinin (2 mg/l)	**Auxin (2 mg/l)**			
	IBA	**NAA**	**IAA**	**2,4-D**
BA	A1	A2	A3	A4
TDZ	A5	A6	A7	A8

3.2.6 Elicitation techniques

3.2.6.1 Preparation of *Trichophyton rubrum* homogenate

The method for preparation of *T. rubrum* homogenate was modified from the method previously described by Rajendran *et al*., (1994). *T. rubrum* was cultured in 500-ml conical flasks containing 200 ml of Sabouraud Dextrose Broth (SDB) at 30^{o}C for 15 days. The concentration of the culture was adjusted by turbidity measurement using spectrophotometer. The absorbance of the culture at 560 nm was adjusted to 0.7 by adding SDB. The culture was homogenized in an ultrasonicator at 100°C for half an hour and then centrifuged at 8000 rpm for 10 min. The supernatant was used as an elicitor after sterilization by autoclaving.

3.2.6.2 Preparation of *Candida albicans* homogenate

The method for preparation of *C. albicans* homogenate was modified from the method previously described by Rajendran *et al*., (1994). *C. albicans* were cultured in 500-ml conical flasks containing 200 ml of SDB on a rotary shaker (110 rpm) at 37°C for 3 days. The concentration of the culture was adjusted by turbidity measurement using spectrophotometer. The absorbance of the culture at 560 nm was adjusted to 1.5 by adding SDB. The culture was homogenized in an ultrasonicator at 100°C for half an hour and then centrifuged at 8000 rpm for 10 min. The supernatant was used as an elicitor after sterilization by autoclaving.

3.2.6.3 Preparation of chitosan stock solution

Chitosan (250 mg) was dissolved in distilled water (10 ml). The solution was adjusted to pH 5.5 with 1N NaOH and the final concentration in cultures was adjusted to 25 mg/ml. Aliquots were autoclaved for 15 min at 121°C prior to use as an elicitor.

3.2.6.4 Preparation of methyl jasmonate stock solution

Methyl jasmonate (11.8 mg) was dissolved in 10 ml of 95% ethanol (v/v) and prepared as a stock solution. Solution was filtered through a membrane-filter (0.2 µm) before being dispensed into the cell suspension cultures at various concentrations (Hwa-Young *et al*., 2008).

3.2.6.5 Effect of elicitor type and concentration on rhinacanthin production

The cell suspension cultures of *R. nasutus* (4-week old) were transferred into B5 liquid medium supplemented with a combination of 1 mg/l BA and 0.5 mg/l IBA. Various types and concentrations of elicitors (*C. albicans* homogenate; 0.5, 1.0, 1.5 and 2.0 % v/v, *T. rubrum* homogenate; 0.5, 1.0, 1.5 and 2.0 % v/v, chitosan; 50, 100, 150, 200 and 250 mg/l and methyl jasmonate; 200, 400, 600, 800 and 1000 µM) were added to the 25-day old cultures. After 3 days incubation with the elicitor, the suspension cultures were harvested and subjected to determination of the rhinacanthin content by HPLC.

3.2.7 Establishment of *R. nasutus* root cultures

3.2.7.1 Effect of explants and light on root formation

The root cultures were either initiated from the whole leaf explants or four-side excised leaf explants of *R. nasutus* on solid B5 medium supplied with 0.1 mg/l IBA. The cultures were incubated at 25 ± 2°C under light or dark conditions. The amounts of roots per explant were recorded after 4 weeks.

3.2.7.2 Effect of light on rhinacanthin production in the root cultures

The root cultures of *R. nasutus* were maintained in B5 liquid medium supplied with 0.1 mg/l IBA. The cultures were incubated on a rotary shaker (80 rpm), at 25 ± 2°C under dark or light conditions. After three successive subcultures, the root cultures (4-week old) were harvested and subjected to HPLC determination of rhinacanthin production.

3.2.7.3 Effect of IBA concentration on root formation

The root cultures were established from the whole leaf explants of *R. nasutus* on solid B5 medium supplied with various IBA concentrations (0.1, 0.5 and 1.0 mg/l). The cultures were at 25 ± 2°C under dark conditions. The amounts of roots per explant were recorded after 4 weeks.

3.2.7.4 Effect of basal medium and IBA concentration on root formation

The root cultures were established from the whole leaf explants of *R. nasutus* on various solid media (B5, MS and WPM) supplied with various IBA concentrations (1.0, 2.0 and 3.0 mg/l). The cultures were incubated at 25 ± 2°C under dark conditions. The amounts of roots per explant were recorded after 4 weeks.

3.2.7.5 Effect of auxin on root formation

The root cultures were established from the whole leaf explants of *R. nasutus* on MS solid media supplied with various types of auxin (IBA, IAA, NAA and 2,4-D) at 3.0 mg/l. The cultures were incubated at 25 ± 2°C under dark conditions. The amounts of roots per explant were recorded after 4 weeks.

3.2.7.6 Effect of kinetin concentration on root formation

The root cultures were established from the whole leaf explants of *R. nasutus* on MS solid media supplied with a combination of 3.0 mg/l IBA and various concentrations of kinetin (0.0, 0.5, 1.0 and 2.0 mg/l), and 30 g/l sucrose. The cultures were incubated at 25 ± 2°C under dark conditions. The amounts of roots per explant were recorded after 4 weeks.

3.2.7.7 Effect of sucrose concentration on root formation

The root cultures were established from the whole leaf explants of *R. nasutus* on MS solid media supplied with 3.0 mg/l IBA and various concentrations of sucrose (30, 60, 90 and

120 g/l). The cultures were incubated at 25 ± 2°C under dark conditions. The amounts of roots per explant were recorded after 4 weeks.

3.2.7.8 Effect semisolid medium on rhinacanthin production

Fifty milligrams of cultured roots were transferred into MS liquid media and MS semisolid media (0.4 % w/v plant agar) supplied with 3.0 mg/l IBA in 250 ml flask. The cultures were incubated on a rotary shaker (80 rpm) at 25 ± 2° C under dark conditions. After three successive subcultures, the root cultures (4-week old) were harvested and subjected to HPLC determination of rhinacanthin production.

3.2.8 HPLC Determination of rhinacanthin production

The dried powder samples (20 mg) were extracted with 20 ml (x2) of ethyl acetate by an aid of ultrasonication for an hour. After the filtrates were evaporated to dryness, the obtained residues were dissolved in methanol (1 ml). These sample preparations were then subjected to analysis of rhinacanthin accumulation using HPLC as described below.

HPLC analysis was carried out using Agilent 1100 series equipped with photodiode-arrays detector and autosampler. Separation was achieved at 25^{o}C on a 150 mm x 4.6 i.d. TSK-gel ODS-80Tm column. The mobile phase consisted of methanol and 5 % aqueous acetic acid (gradient evolution as follow 0 -10 min; 85:15, 17 - 30 min; 90 -10) and was pumped at a flow rate of 0.4 ml/min. The injection volume was 20 µl. The quantitative wavelength was set at 254 nm. The rhinacanthin-C, -D and -N were determined using HPLC. Peak retention times and UV absorption spectra of the corresponding peaks compared with the authentic compounds were used for identification of the rhinacanthin formation. For quantitative analysis of rhinacanthin content in samples of *R. nasutus*, the area under the peaks of each rhinacanthin were recorded and converted to concentration using their calibration curves. The calibration curves were established from the standards rhinacanthin-C, -D and -N at the concentration between 3.60 - 57.5 µg/ml, 0.24 - 7.69 µg/ml and 0.16 - 10.0 µg/ml, respectively.

3.2.9 Time course of growth and rhinacanthin production of *R. nasutus* root cultures

Fifty milligrams of the cultured roots were transferred into MS semisolid medium in 250 ml flask and cultured on a rotary shaker (80 rpm) at 25 ± 2°C, under dark conditions. The root cultures (3 flasks) were harvested every 5 days for a period of 30 days. The dry weights were recorded after drying at 50°C for 24 hours. The content of rhinacanthin was determined by the HPLC method as described in the sections 3.2.8. These data were then plotted to obtain growth and rhinacanthin production curves.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Establishment of callus cultures

The ability of the young leaf explants of *R. nasutus* to form callus was investigated by manipulation of BA and IBA composition in solid B5 medium. The callus formation was observed after 6 weeks of the initiation. The callus formation was graded in 5 levels as follows; 0 = none, 1 = least, 2 = little, 3 = medium, 4 = much 5 = very much. It was found that B5 medium supplied with a combination of 2.0 mg/l BA and various concentrations of IBA (0.1, 0.5, 1.0 and 2.0 mg/l) were capable of inducing the callus formation. All calli appearances were dense with a yellowish color (Fig. 4.1). In addition, IBA concentration plays an important role on callus formation of *R. nasutus*. An increasing of IBA concentration in the culture medium, an increasing of callus formation was observed. At the concentration of BA 2.0 mg/l, the optimum concentration of IBA for the callus formation was 0.5 mg/l (Table 4.1).

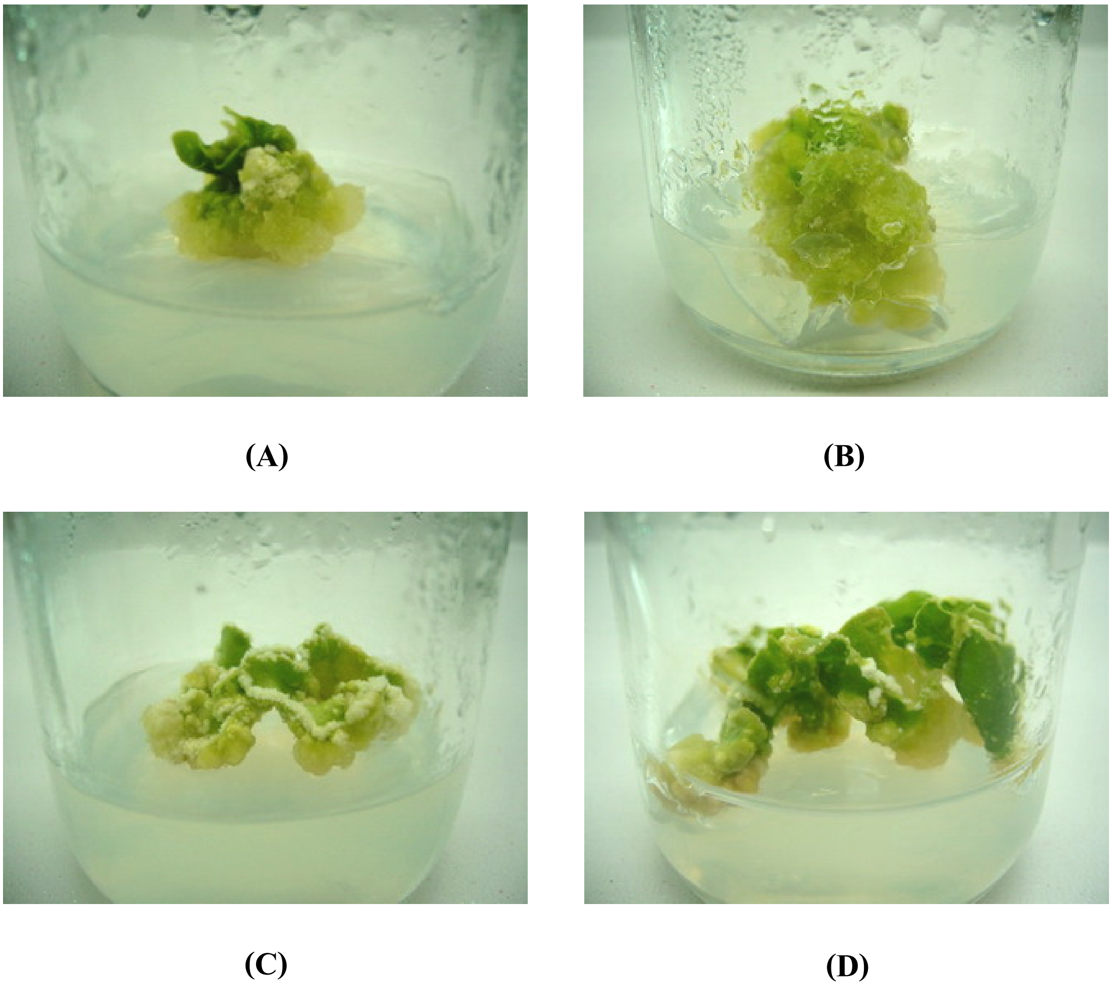


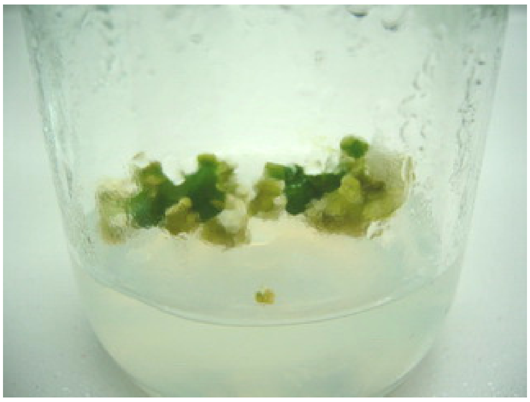
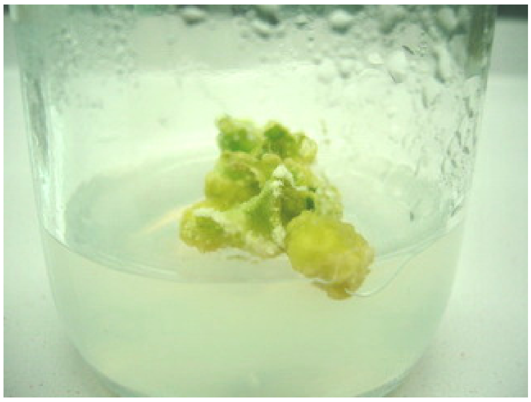
Figure 4.1 *R. nasutus* callus culture initiated on solid B5 medium supplied with 2.0 mg/l BA and 0.1 mg/l IBA (A); 2.0 mg/l BA and 0.5 mg/l IBA (B); 2.0 mg/l BA and 1.0 mg/l IBA (C); and 2.0 mg/l BA and 2.0 mg/l IBA (D)

Table 4.1 Effect of IBA concentration on callus formation of *R. nasutus*

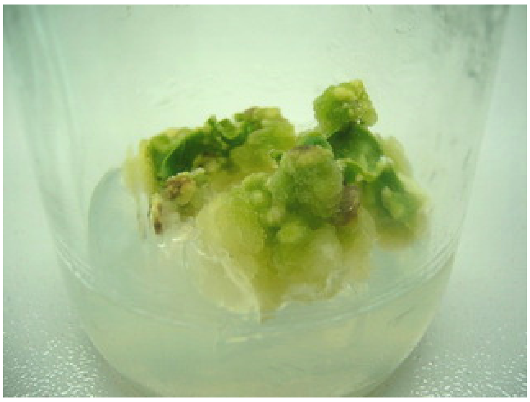
Plant growth regulators	**Color of callus**	**Characteristics of callus**	**Level of callus formation***
2.0 mg/l BA + 0.1 mg/l IBA	Yellowish	Dense	3
2.0 mg/l BA + 0.5 mg/l IBA	Yellowish	Dense	5
2.0 mg/l BA + 1.0 mg/l IBA	Yellowish	Dense	2
2.0 mg/l BA + 2.0 mg/l IBA	Yellowish	Dense	2

* 0 = none, 1 = least, 2 = little, 3 = medium, 4 = much 5 = very much

The callus formation of *R. nasutus* was further examined by variation of BA concentrations (0.1, 0.5, 1.0 and 2.0 mg/l) with a fixed concentration of IBA at 0.5 mg/l in B5 medium. The appearances of all calli were the same as those from B5 medium supplied with a combination of 2.0 mg/l BA and 0.5 mg/l IBA (Fig. 4.2). An increasing of BA concentration in the culture medium resulted in an increasing of callus formation (Table 4.2). Thus, the most appropriate concentrations of BA and IBA for callus induction of *R. nasutus* were 2.0 and 0.5 mg/l, respectively. The callus could be maintained by periodic subculture into the same solid B5 medium at 6-week intervals.

(A) (B)

(C) (D)

Figure 4.2 *R. nasutus* callus culture initiated on solid B5 medium supplied with 0.5 mg/l IBA and 0.1 mg/l BA (A); 0.5 mg/l IBA and 0.5 mg/l BA (B); 0.5 mg/l IBA and 1.0 mg/l BA (C); and 0.5 mg/l IBA and 2.0 mg/l BA (D)

Table 4.2 Effect of BA concentration on callus formation of *R. nasutus*

Plant growth regulators	Color of callus	Characteristics of callus	Level of callus formation*
0.5 mg/l IBA + 0.1 mg/l BA	Yellow-Green	Dense	1
0.5 mg/l IBA + 0.5 mg/l BA	Yellow-Green	Dense	1
0.5 mg/l IBA + 1.0 mg/l BA	Yellow-Green	Dense	3
0.5 mg/l IBA + 2.0 mg/l BA	Yellow-Green	Dense	5

* 0 = none, 1 = least, 2 = little, 3 = medium, 4 = much 5 = very much

4.2 Establishment of *R. nasutus* cell suspension cultures

The cell suspension cultures of *R. nasutus* were initiated by transferring the callus into liquid B5 medium containing 2.0 mg/l BA and 0.5 mg/l IBA. The cell suspension cultures were maintained in a rotary shaken flask at 150 rpm under dark conditions. The suspension cultures appeared to be homogeneous with yellowish color. After several subcultures, the stable cell suspension cultures were subjected to determination of rhinacanthin production by the HPLC method as described in the section 3.2.8. Unfortunately, it was found that the cell suspension cultures did not accumulate any rhinacanthin (Fig 4.3). This may be due to a dedifferentiation of the cells. Dedifferentiation is usually accompanied by an apparent loss of their ability to accumulate secondary metabolites. The reason may be a lack of gene expressions that control the essential steps of the biosynthetic pathway in non-specialize cells or the non-ability of storage site or an unregulated catabolism of secondary product (Charlwood and Rhodes, 1990).

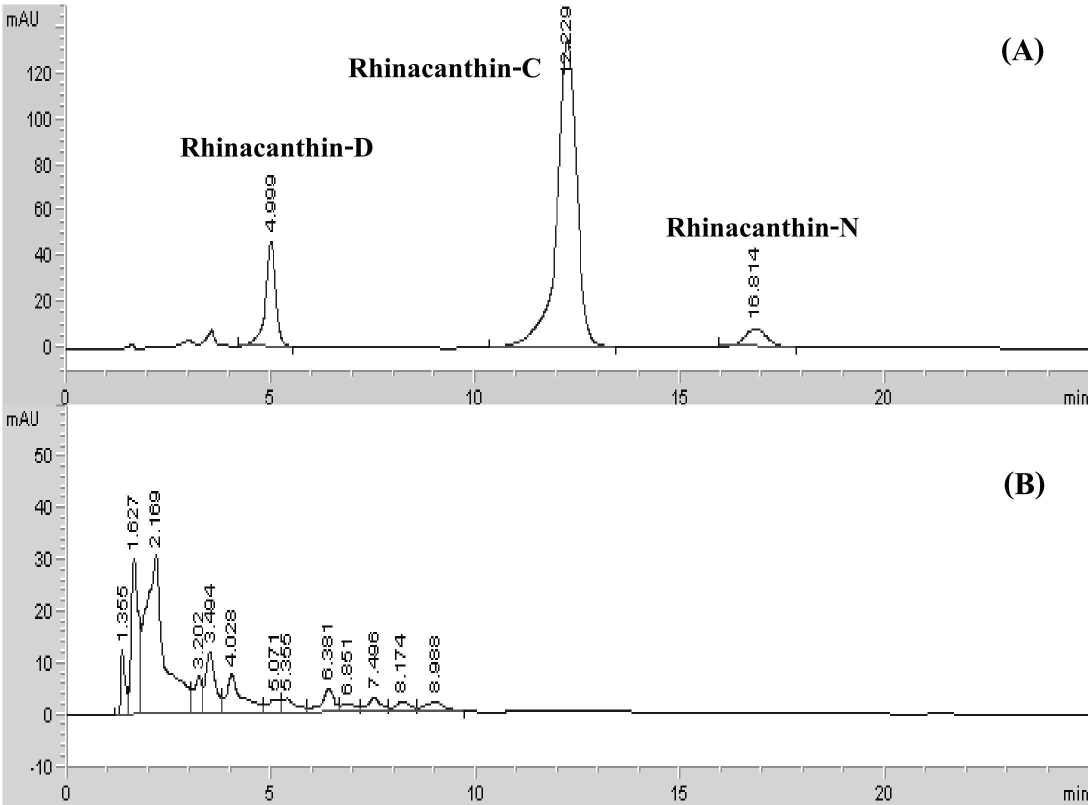


Figure 4.3 HPLC-chromatograms of the standard rhinacanthins (A) and extracts of the cell suspension cultures (B)

4.3 Induction of rhinacanthin production by medium manipulation

Although the cell suspension cultures were established in B5 medium supplied with 2.0 mg/l BA and 0.5 mg/l IBA, the cells lose their ability to produce rhinacanthins. Medium manipulation was therefore examined in order to induction of rhinacanthin formation in *R. nasutus* cell suspension cultures. The effects of auxin and cytokinin on rhinacanthin production of *R. nasutus* cell suspension cultures were determined. Four types of auxin (NAA, 2,4-D, IBA and IAA) and two types of cytokinin (BA and TDZ) at the concentration of 2.0 mg/l were examined. The results showed that neither of the cell suspension cultures was capable of producing rhinacanthins. However, a combination of 2.0 mg/l BA and 2.0 mg/l IBA in B5 medium produced more homogenous cell suspension cultures than the other plant growth regulator combinations. Thus, medium manipulation was further examined by variation of IBA concentrations (0.1, 0.5, 1.0 and 2.0 mg/l) with a combination of 2.0 mg/l BA. After 4 weeks, the cell suspension cultures were harvested and subjected to determination of rhinacanthin accumulation by HPLC. The results also showed that neither of the cell suspension cultures was capable of producing rhinacanthins. However, a combination of 2.0 mg/l BA and 0.5 mg/l IBA in B5 medium produced more homogenous cell suspension cultures than the other combinations. Thus, the effect of BA concentrations on induction of rhinacanthin accumulation was further examined by variation of BA concentrations (0.1, 0.5, 1.0 and 2.0 mg/l) with a fixed concentration of BA at 0.5 mg/l in B5 medium. After 4 weeks, the cell suspension cultures were harvested and subjected to determination of rhinacanthin accumulation by HPLC. The results also showed that neither of the cell suspension cultures was capable of producing rhinacanthins. However, a combination of 1.0 mg/l BA and 0.5 mg/l IBA in B5 medium produced more homogenous cell suspension cultures (Fig. 4.4) than the other combinations. Our plant growth regulator manipulation fails to induce rhinacanthin formation in *R. nasutus* cell suspension cultures. The result implies that rhinacanthins can not accumulate in the cell suspension culture system of *R. nasutus*. This may be due to lacks of enzyme involves in the biosynthesis of rhinacanthins or cell compartment for rhinacanthin accumulation in the cell suspension cultures of *R. nasutus*. However, the cell suspension cultures were maintained in B5 medium supplied with 1.0 mg/l BA and 0.5 mg/l IBA and used in elicitation experiments.

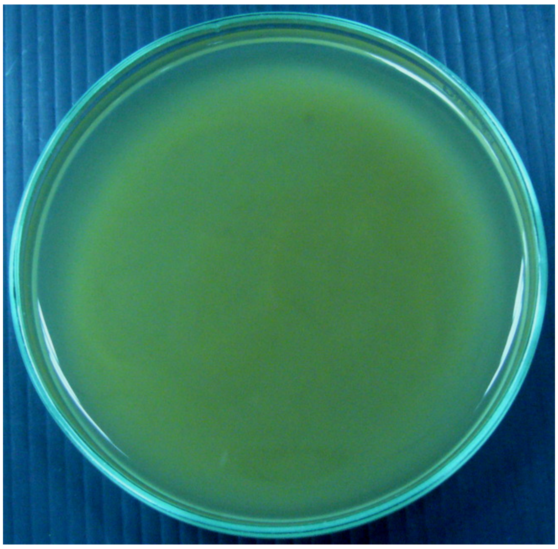


Figure 4.4 *R.nasutus* cell suspension culture maintained in B5 medium supplied with 1.0 mg/l BA and 0.5 mg/l IBA

4.4 Induction of rhinacanthin production by elicitation

The elicitation technique was further examined for induction of rhinacanthin production in *R. nasutus* cell suspension cultures. Two types of the elicitor, including complex elicitor preparation (*C. albicans* homogenate and *T. rubrum* homogenate) and chemical defined elicitor (chitosan and methyl jasmonate) were used in this study. The effect of the elicitor concentrations on rhinacanthin production was also determined. Unfortunately, neither elicitors used in this study stimulated rhinacanthin formation in *R. nasutus* cell suspension cultures. Although it has been reported that elicitation technique successfully induced naphthoquinone production in *Plumbago rosea* (plumbagin), *Lithospermum erythrorhizon* (shikonin), *Arnebia euchroma* (shikonin) and *Drosera capensis* (7-methyl juglone) cell suspension culture (Komaraiah *et al*., 2002; Kim *et al*., 1990; Fu and Lu, 1999 and Ziaratnia *et al*., 2009), this technique could not induce naphthoquinone (rhinacanthins) production in *R. nasutus* cell suspension cultures.

4.5 Establishment of *R. nasutus* root cultures

4.5.1 Effect of explants and light on root formation

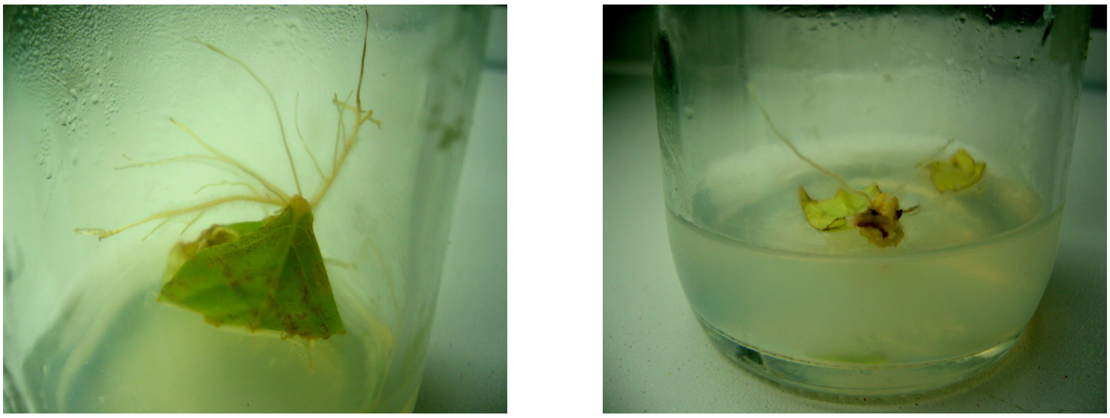
Establishment of *R. nasutus* root cultures using either four-side excised leaves or the whole leaves as the starting materials on the solid B5 medium supplied with 0.1 mg/l IBA and cultured under light or dark conditions showed that only the explants that initiated under the dark conditions were capable of producing the root cultures (Table 4.3). In addition, the whole leaf explants produced higher amount of roots than the four-side excised leaf explant. The root formation on the whole leaf explants was 10 times higher than that on four-side excised leaf explants.

The root formation of *R. nasutus* was usually take place at the middle vein on the leaf base (Fig. 4.5). This may be the reason why the four-side excised leaf explants produced fewer amounts of the roots. Moreover, the root formation of *R. nasutus* is also inhibited by light. However, the effect of light on promotion of root formation has also been reported (Lovell and Moore, 1969). Thus, the whole leaf explants that incubated under dark conditions were appropriate for production of *R. nasutus* root cultures.

Table 4.3 Effect of the leaf explants and light on root formation of *R. nasutus*

Type of leaf explants	Culture conditions	Number of root per explant (Mean ± S.E.)
Four-side excised leaves	Light (n = 10)	0
	Dark (n = 10)	0.4 ± 0.84
Whole leaves	Light (n = 10)	0
	Dark (n = 10)	4 ± 2.05*

* Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$.



(A) (B)

Figure 4.5 *R. nasutus* root cultures initiated from the whole leaf explants (A) and the four-side excised leaf explants (B) under dark conditions

4.5.2 Effect of light on rhinacanthin production in the root cultures

After 4 weeks, the root cultures were transferred to the same B5 liquid medium and cultured either under light or dark conditions. After several subcultures, the cultured roots (4-week old) were subjected to HPLC quantitative determination of rhinacanthin. The identity of rhinacanthin-C, -D and -N Peak in the HPLC chromatograms were confirmed by comparing their UV absorption spectra with those of the authentic compounds (Fig 4.6, 4.7 and 4.8, respectively). For quantitative analysis of rhinacanthin content various *R. nasutus* extracts, the area under the peak of each rhinacanthins was converted to concentration using its calibration curve as shown in figure 4.9, 4.10 and 4.11, respectively. The calibration curves were established from the standards rhinacanthin-C, -D and -N at the concentration between 3.60 - 57.5 µg/ml, 0.24 - 7.69 µg/ml and 0.16 - 10.0 µg/ml, respectively. The linear equations of Y = 106504X + 21.141 (r^2 = 1.0000), Y = 178576X + 1.5311 (r^2 = 1.0000) and Y = 276858X + 8.2617 (r^2 = 0.9999) correspond to rhinacanthin-C, -D and -N, respectively.

On the basis of HPLC analysis, the cultured roots in both conditions produced only rhinacanthin-C as the major naphthoquinone (Fig. 4.12). However, the root cultures in the dark conditions produced higher biomass and amount of rhinacanthin-C (2.80 ± 0.009 mg/g DW) than that of in the light conditions (0.68 ± 0.011 mg/g DW) as shown in Table 4.4. This result agrees with the previous report on the accumulation of rhinacanthins, which higher accumulated in the roots of the intact plant than the aerial parts that exposed to light (Panichayupakaranant *et al*., 2006). Moreover, the result demonstrates that both growth and rhinacanthin-C formation of *R. nasutus* cultures were inhibited by light. Thus, the root cultures that incubated under dark conditions were appropriate for growth and rhinacanthin-C production of *R. nasutus* cultures.

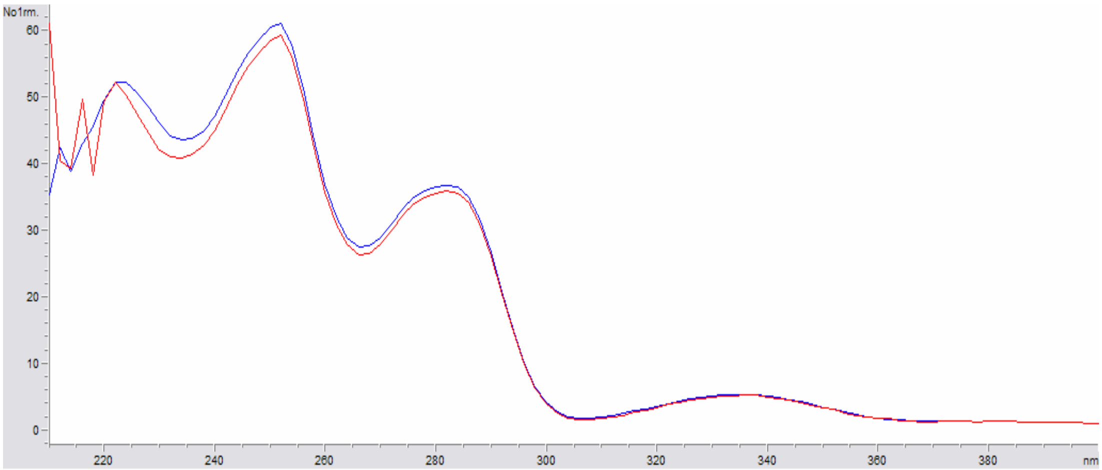


Figure 4.6 UV absorption spectra of authentic rhinacanthin-C (blue line) and the compound of similar RT obtained from R. nasutus tissue culture extracts (red line). UV spectra were recorded using the HPLC-UV diode array detector.

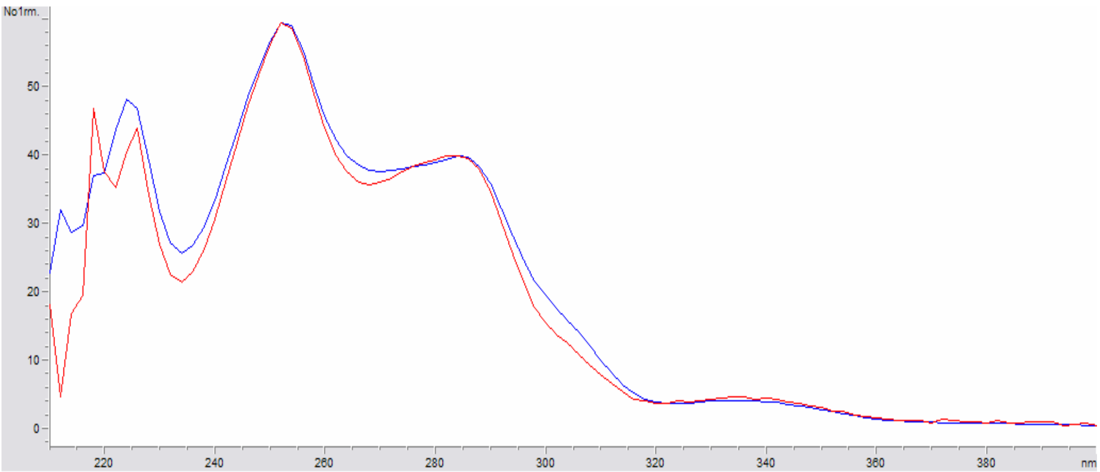


Figure 4.7 UV absorption spectra of authentic rhinacanthin-D (blue line) and the compound of similar RT obtained from *R. nasutus* tissue culture extracts (red line). UV spectra were recorded using the HPLC-UV diode array detector.

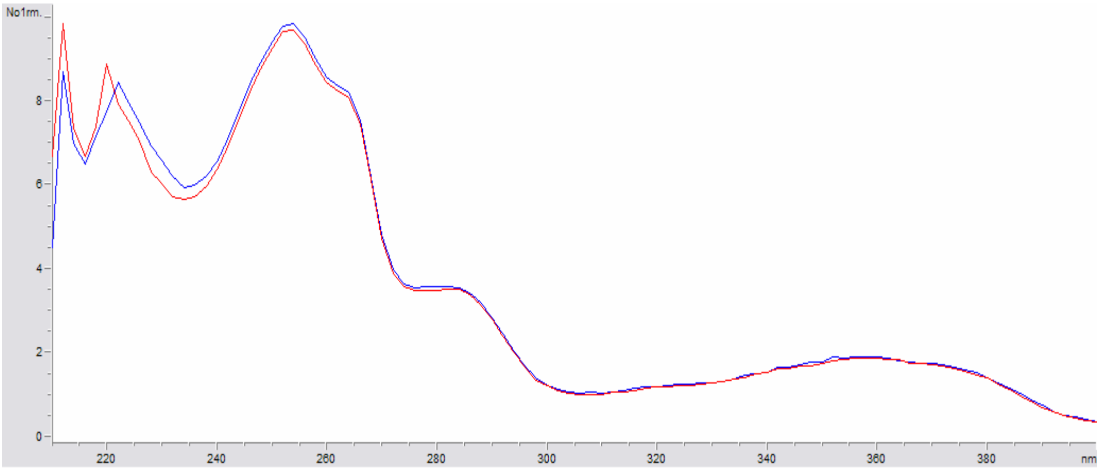


Figure 4.8 UV absorption spectra of authentic rhinacanthin-N (blue line) and the compound of similar RT obtained from *R. nasutus* tissue culture extract (red line). UV spectra were recorded using the HPLC-UV diode array detector.

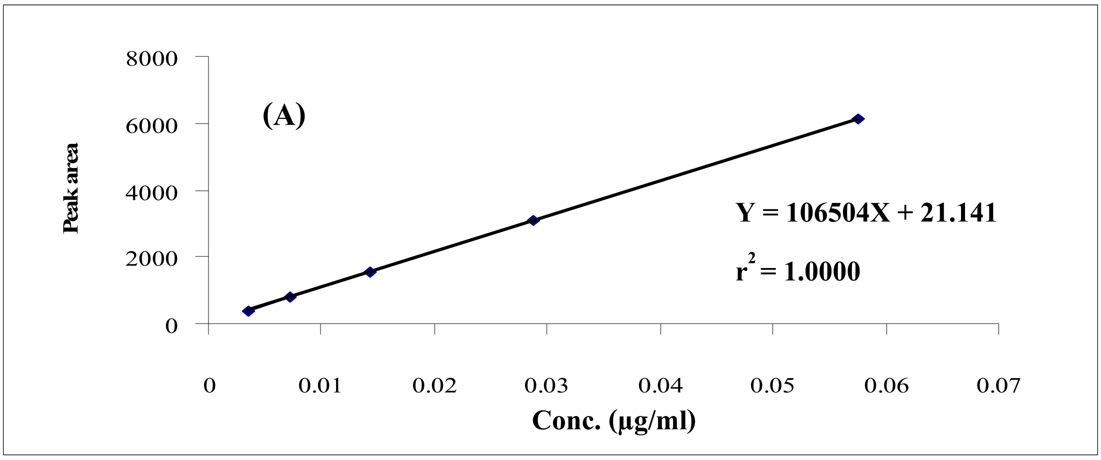


Figure 4.9 Standards curve of rhinacanthin-C

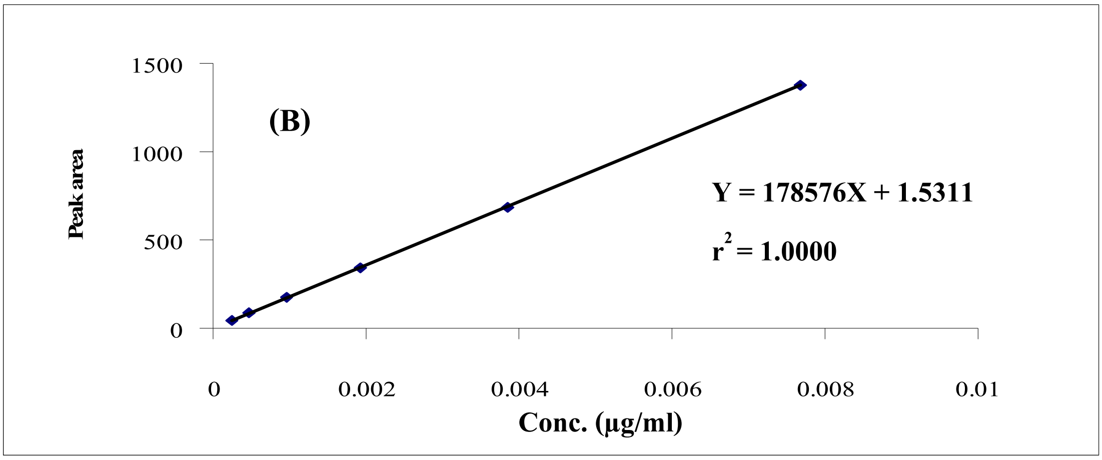


Figure 4.10 Standards curve of rhinacanthin-D

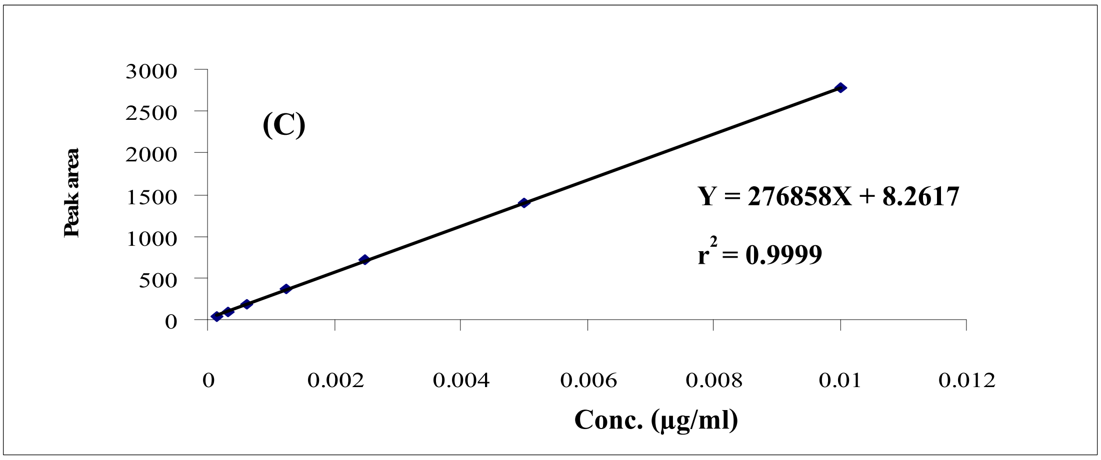


Figure 4.11 Standards curve of rhinacanthin-N

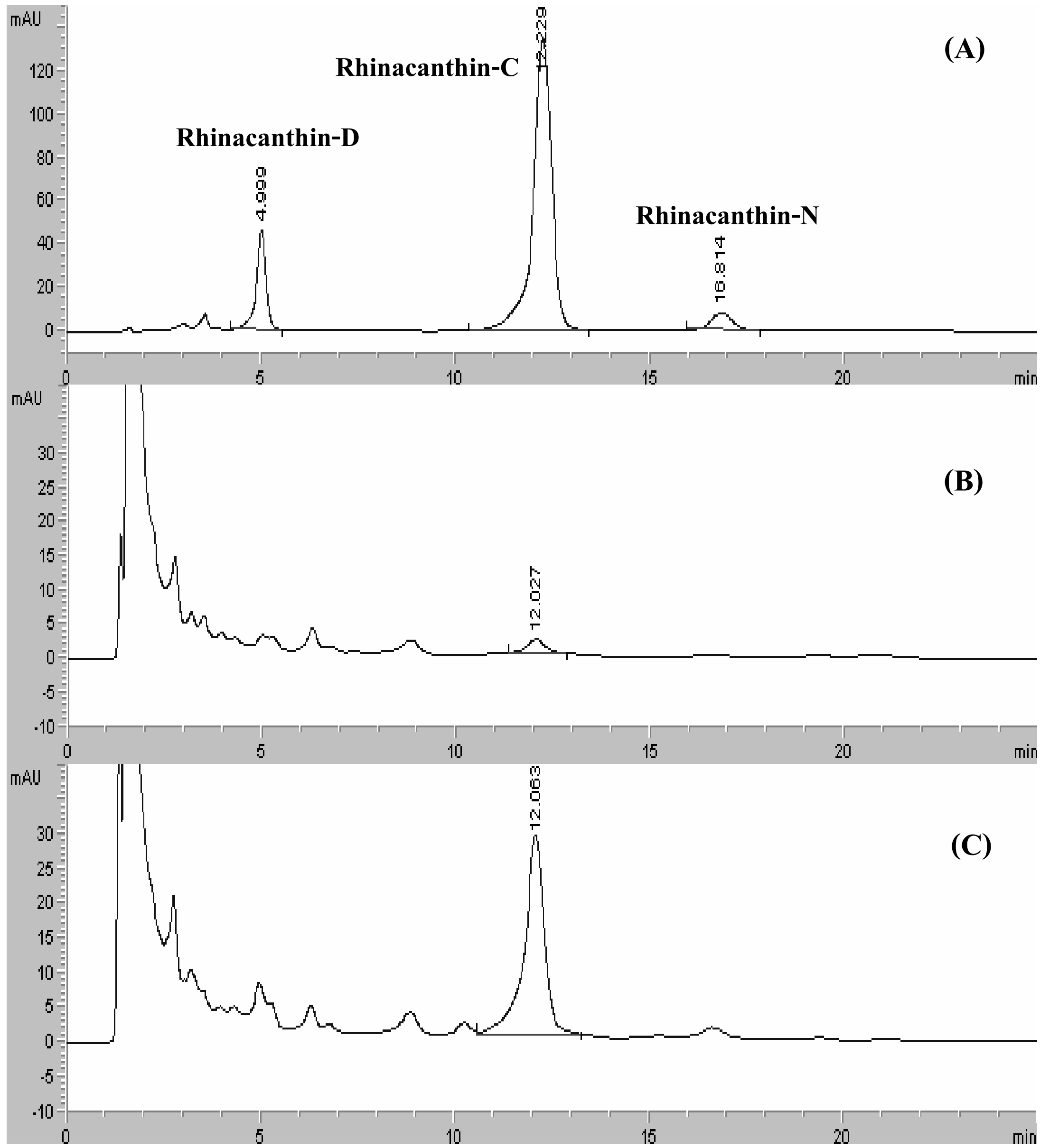


Figure 4.12 HPLC-chromatograms of the standard rhinacanthins (A), extracts of the root cultures incubated under light conditions (B); and dark conditions (C)

Table 4.4 Effect of light on growth and rhinacanthin-C production in *R. nasutus* root cultures

Culture conditions	Dry biomass (mg/ 250-ml flask ± S.E.)	Rhinacanthin-C content (mg/g DW ± S.D.)
Light	3.2 ± 0.26	0.68 ± 0.011
Dark	7.3 ± 0.20*	2.80 ± 0.009*

* Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$. ($n = 3$)

4.5.3 Effect of cultured media on root formation of *R. nasutus*

Although the root cultures were established on B5 medium supplied with 0.1 mg/l IBA and 20 g/l sucrose, their growth rate were very slowly. Medium manipulation was therefore examined in order to improve growth of *R. nasutus* root cultures. In this study, the amounts of root formations per explant were used as growth parameter. Variation of IBA concentrations (0.1, 0.5 and 1.0 mg/l) in B5 medium found that the root formation was increased as IBA concentration dependent. The root formation of *R. nasutus* was shown in figure 4.13. The highest root formation was found at 1.0 mg/l IBA (Table 4.5).

Figure 4.13 *R. nasutus* root culture initiated in solid B5 medium supplied with 1.0 mg/l IBA

Table 4.5 Effect of IBA concentration on root formation of *R. nasutus*

IBA concentration (mg/l)	Number of root per explant (Mean ± S.E.)
0.1	3.9 ± 2.18
0.5	4.1 ± 1.52
1.0	5.2 ± 2.52*

* Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$. ($n = 10$)

The further medium manipulation was therefore performed by variation of the basal medium (B5, MS and WPM) as well as IBA concentrations (1.0, 2.0 and 3.0 mg/l). The root formation of *R. nasutus* was showed in figure 4.14. The result showed that MS medium supplied with 3.0 mg/l IBA was the most appropriate cultured medium for root formation of *R. nasutus* (Table 4.6).

Figure 4.14 *R. nasutus* root culture initiated in solid MS medium supplied with 3.0 mg/l IBA

Table 4.6 Effect of culture medium and IBA concentration on root formation of *R. nasutus*

Type of medium salts formula	**IBA concentration (mg/l)**	**Number of root per explant (Mean ± S.E.)**
B5	1.0	5.7 ± 2.31
	2.0	6.0 ± 2.53
	3.0	6.9 ± 1.79
MS	1.0	7.2 ± 2.09
	2.0	8.0 ± 3.43
	3.0	14.4 ± 2.63*
WPM	1.0	2.7 ± 2.05
	2.0	3.5 ± 2.36
	3.0	6.0 ± 2.90

* Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$. (n = 10)

To determine the effect of auxin type on the root formation, medium manipulation was further performed by variation of auxin (IBA, IAA, NAA and 2,4-D) at 3.0 mg/l in MS medium. It was found that the most appropriate auxin for the root formation of *R. nasutus* was 3.0 mg/l IBA (Table 4.7). In contrast, high concentration of 2,4-D completely inhibited the root formation.

Table 4.7 Effect of type of auxin on root formation of *R. nasutus*

Type of auxin	**IBA concentration (mg/l)**	**Number of root per explant (Mean ± S.E.)**
IBA	3.0	12.13 ± 1.355*
IAA	3.0	1.60 ± 1.594
NAA	3.0	1.53 ± 1.641
2,4-D	3.0	0

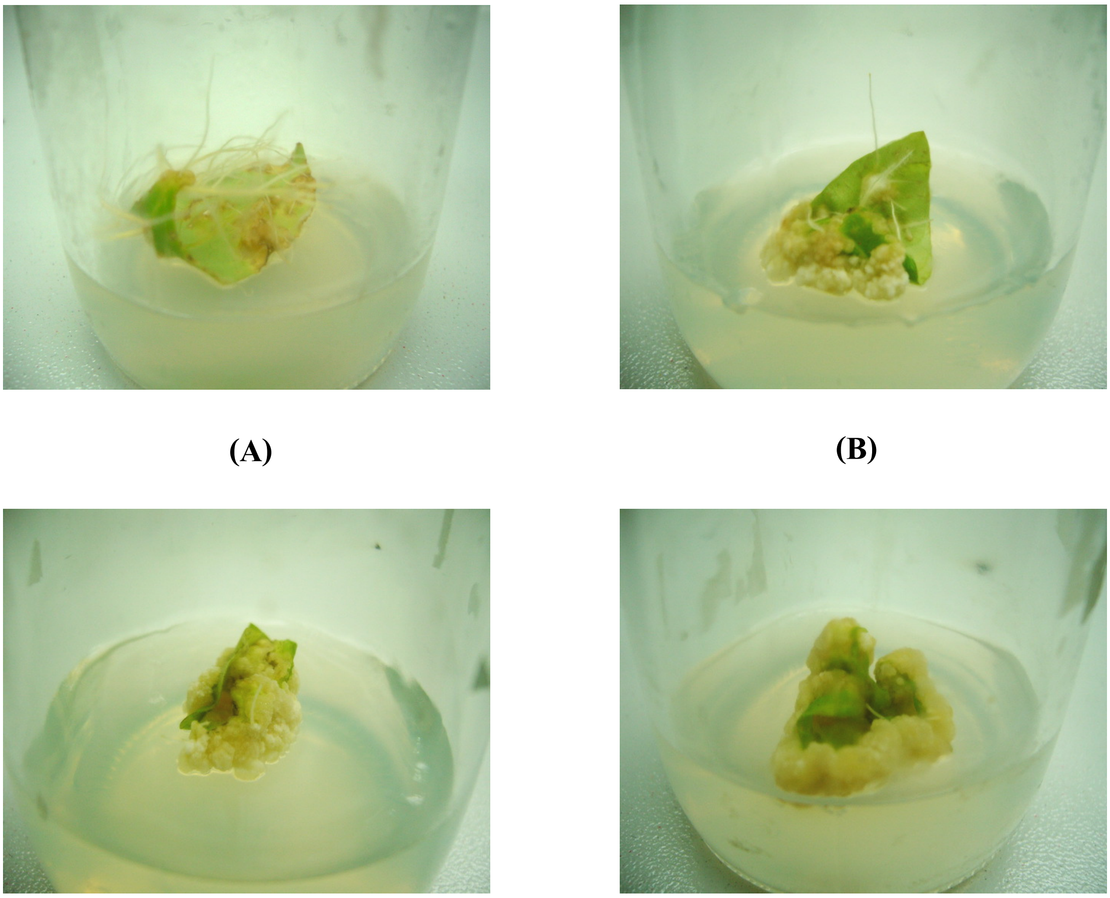
* Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$. (n = 15)

It has been reported that cytokinin also an important role on root formation of *I. balsamina* (Panichayupakaranant and De-Eknamkul, 1992). We therefore determined the effect of kinetin concentrations (0.0, 0.5, 1.0 and 2.0 mg/l) in MS medium supplied with 3.0 mg/l IBA on the root formation of *R. nasutus*. The resulted indicated that an increasing of kinetin concentration in the cultured medium a decreasing of root formation was observed (Table 4.8). Moreover, an increasing of kinetin concentration resulted in dedifferentiation of the root cultures. Callus formation was observed at the concentration of kinetin as shown in figure 4.15. This suggests that kinetin inhibits root formation of *R. nasutus*.

Table 4.8 Effect of kinetin concentration on root formation of *R. nasutus*

Kinetin concentration (mg/l)	**Number of root per explant (Mean ± S.E.)**
0	12.0 ± 1.41*
0.5	5.41 ± 1.72
1.0	2.08 ± 1.62
2.0	0.75 ± 1.05

* Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$. ($n = 12$)



(A) (B) (C) (D)

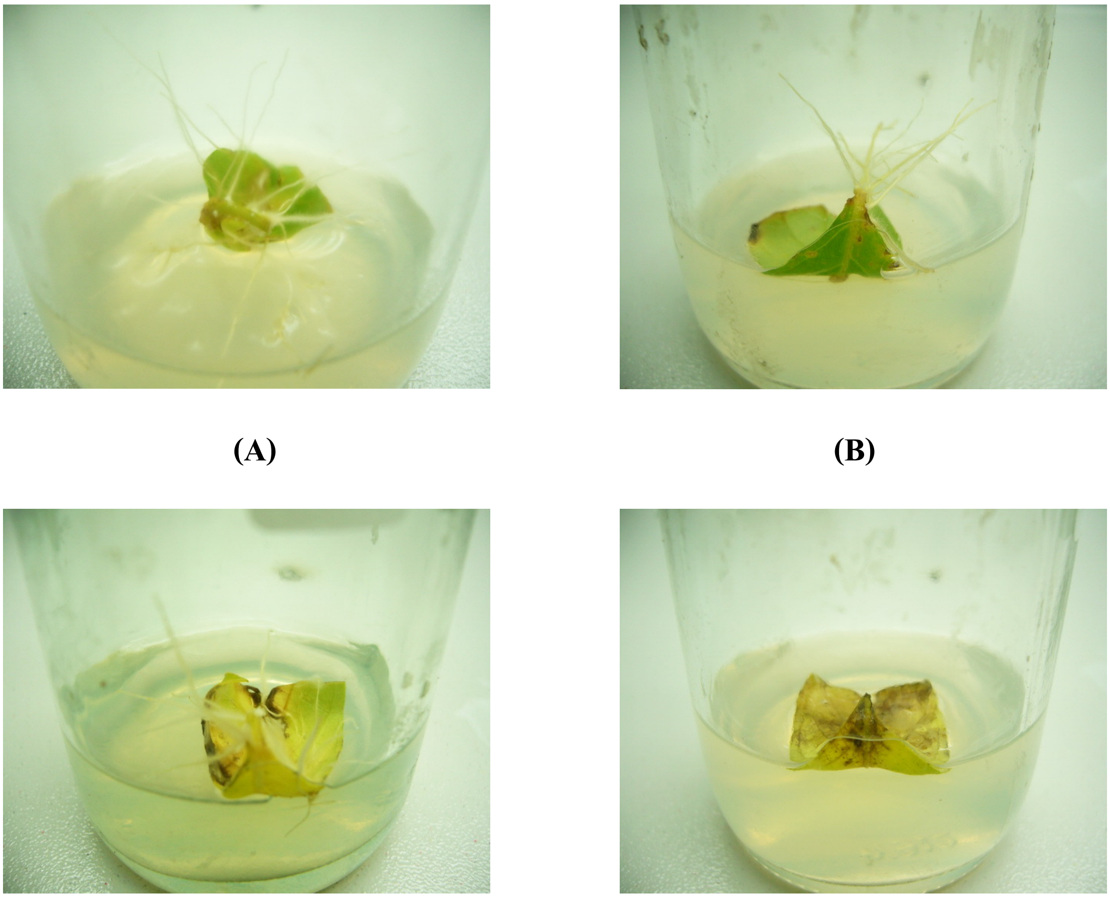
Figure 4.15 *R. nasutus* root cultures initiated in solid MS medium supplied with 3.0 mg/l IBA (A); 3.0 mg/l IBA and 0.5 mg/l Kn (B); 3.0 mg/l IBA and 1.0 mg/l Kn (C); and 3.0 mg/l IBA and 2.0 mg/l Kn (D)

Sucrose is one of the most important nutrients for plant *in vitro* culture. It is known as a carbon source that essential for plant cell growth. In this study, variation of sucrose concentrations (30, 60, 90 and 120 g/l) was also examined to determine the effect on the root formation of *R. nasutus*. The results exhibited that an increasing of sucrose concentration in the cultured medium a decreasing of root formation was observed (Table 4.9). This may be due to higher osmotic pressure of the cultured medium. An increasing of sucrose concentrations results in an increasing of the osmotic of the culture medium. Higher osmotic pressure may inhibit nutrient and plant growth regulator absorption into plant cells. The root formation was therefore decrease when increase the sucrose concentration as shown in figure 4.16. These medium manipulation studies suggests that MS medium supplied with 3.0 mg/l IBA and 30 g/l sucrose was the most appropriate for root formation of *R. nasutus*.

Table 4.9 Effect of sucrose concentration on root initiation of *R. nasutus*

Sucrose concentration (g/l)	**Number of root per explant (Mean ± S.E.)**
30	11.2 ± 1.30*
60	5.4 ± 0.54
90	4.8 ± 0.83
120	0

* Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$. (n = 10)



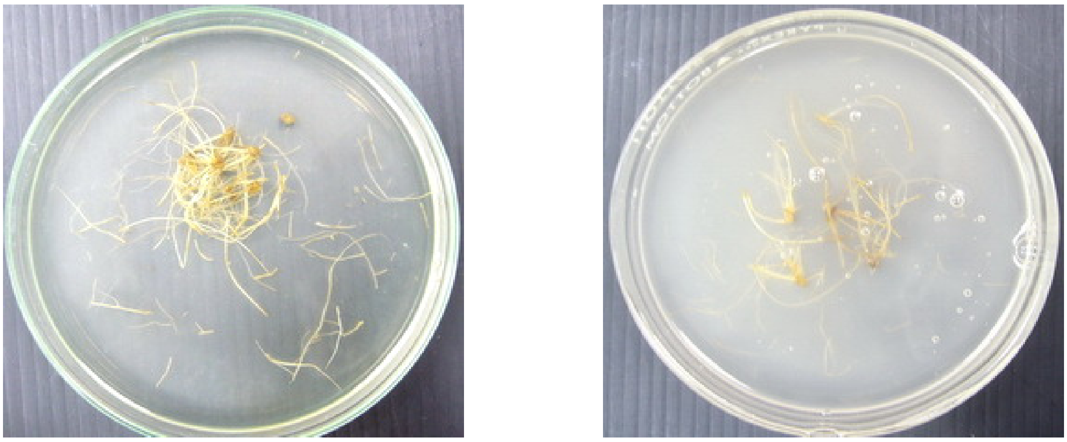
(A) **(B)**

(C) **(D)**

Figure 4.16 *R. nasutus* root cultures initiated in solid MS medium supplied with 3.0 mg/l IBA and 30 g/l sucrose (A); 60 g/l sucrose (B); 90 g/l sucrose (C) and 120 g/l sucrose (D)

4.5.4 Effect of semisolid medium on rhinacanthin production

The root cultures of *R. nasutus* were then maintained in MS medium supplied with 3.0 mg/l IBA and 30 g/l sucrose (Fig. 4.17A). After several subcultures, the root cultures (4-week old) were subjected to quantitative determination of rhinacanthin production. On the basis of HPLC analysis, the root cultures produced only rhinacanthin-C (Fig. 4.18B). In addition, accumulation of rhinacanthin in the root cultures was very low (0.03 mg/g DW). The rhinacanthin formation was lower than that of the former root cultures in B5 medium supplied with 0.1 mg/l IBA. This may be due to a better growth of the root cultures in MS liquid medium supplied with 3.0 mg/l IBA was capable of decreasing rhinacanthin production (Table 4.10) . An improving of rhinacanthin production in the root cultures was therefore examined by mimic immobilization techniques.



(A) **(B)**

Figure 4.17 *R. nasutus* root cultures in liquid MS medium (A) and semisolid MS medium (B) supplied with 3.0 mg/l IBA and 30 g/l sucrose

Immobilization technique with the calcium alginate gel has successfully increased naphthoquinone production in *Plumbago rosea* (Komaraiah *et al*., 2003). In this study, the cultured root were transferred into the MS medium containing 4.0 g/l agar and maintained as semisolid culture on the shaker (80 rpm) (Fig. 4.17B). After several subcultures, the root cultures (4-week old) were harvested and subjected to determination of rhinacanthin production by HPLC. In the semisolid conditions, the root cultures were capable of producing rhinacanthin-C and -D as well as increasing their production with the content of rhinacanthin-C and -D was 0.72 and 0.02 mg/g DW, respectively (Fig. 4.18C and Table 4.10). Our finding suggests that culturing in semisolid medium may be a strategy for improving of secondary metabolite production in plant tissue cultures.

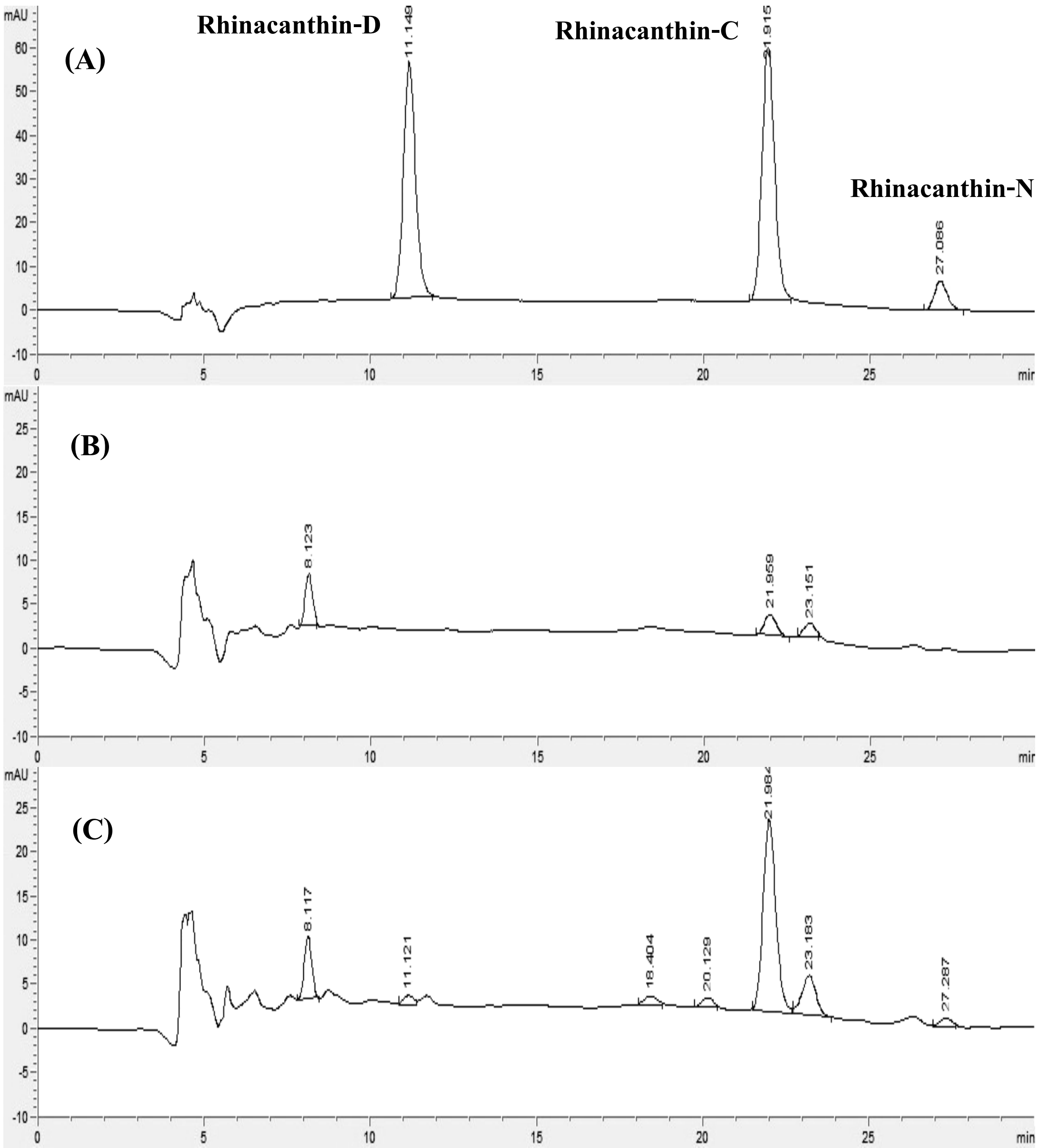


Figure 4.18 HPLC-chromatograms of the standard rhinacanthin (A); extracts of root culture in liquid MS medium (B) and extracts of root culture in semisolid MS medium (C)

Table 4.10 Rhinacanthin content in *R. nasutus* roots cultured in liquid and semisolid MS (4 wks)

MS medium	Dried weight (mg/flask ± S.E.)	Rhinacanthin content (mg/g DW ± S.D.)	
		Rhinacanthin-C	Rhinacanthin-D
Semisolid	9.13 ± 0.321	0.72 ± 0.008	0.02 ± 0.000
Liquid	15.53 ± 0.808	0.03 ± 0.001	n.d.

n.d. : can not calculated due to the area under the peak is under the lower limit of detection (n = 3)

4.6 Time course of growth and rhinacanthin production by *R. nasutus* root cultures

The relationship between growth and rhinacanthin production of *R. nasutus* root cultures during a period of 30 days were examined in this study. The dry weight of the harvested root biomass was used as a parameter for expression the culture growth (Table 4.11). The result showed that the growth pattern of *R. nasutus* appears to be a normal sigmoid curve (Fig 4.19). The root cultures spend a period of ten days for cell adaptation in a lag phase. To overcome this long period of the lag phase, an increase of the initial biomass should be carried out. After the root cultures have adapted to the fresh medium, their growth was gradually increased. This results in a continuous increase in the biomass throughout the period of 15 days. The cultures growth then reached a stationary phase at days 25. The highest value of the biomass obtained was 9.3 mg/flask, which were equivalent to about two times of the inoculated cell culture biomass.

Table 4.11 Time course of growth of *R. nasutus* root cultures

Days	Dried weight (mg/flask ± S.E.)
0	4.83 ± 0.602
5	4.76 ± 0.251
10	4.80 ± 0.530
15	7.66 ± 0.740
20	8.56 ± 0.503
25	9.26 ± 0.351
30	9.33 ± 0.420

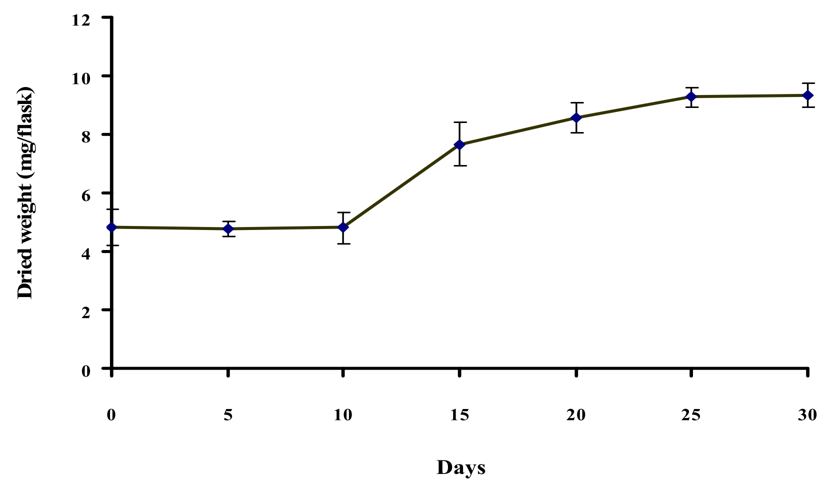


Figure 4.19 Time course of growth of *R. nasutus* root cultures

The time course of rhinacanthin-C, -D and -N productions showed that there was a fluctuation of rhinacanthin-D accumulation during the growth cycle of the root cultures. The fluctuation of rhinacanthin-D production in day 0 and day 10 may be come from a technical error of quantitative analysis. However, all rhinacanthin productions in the root cultures seem to be started after the lag phase (after 10 days). Rhinacanthin production was found to increase with its highest rhinacanthin accumulation at the linear phase (day 15) of the growth cycle (Fig. 4.20, 4.21 and 4.22). This suggests that the biosynthesis of rhinacanthin-C, -D and -N take place at the same time as production of other primary metabolites used for growth promotion. This phenomenon is different from most secondary metabolites productions, which usually take place when the growth rate begins to decline (Dixon, 1991). The result suggests that the suitable period of the root culture harvesting is 15 days after subculture. Although the root cultures of *R. nasutus* were capable of producing rhinacanthin-C, -D and -N (Fig. 4.23), their content still lower than those of the leaves and roots of the intact plants (Table 4.13). Therefore, further studies should be focusing on improving of growth and rhinacanthin production of *R. nasutus* root cultures.

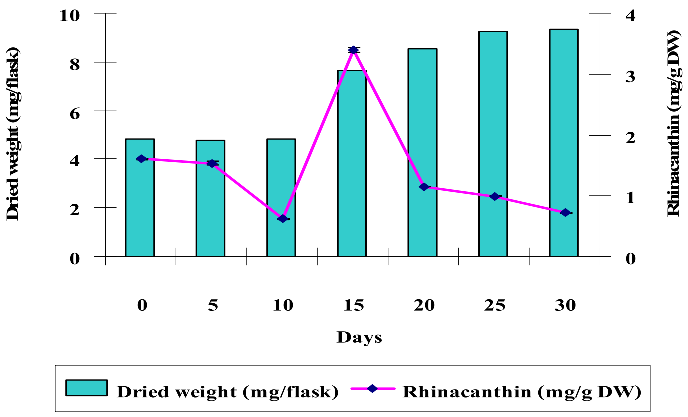


Figure 4.20 Time course of rhinacanthin-C production in *R. nasutus* root cultures

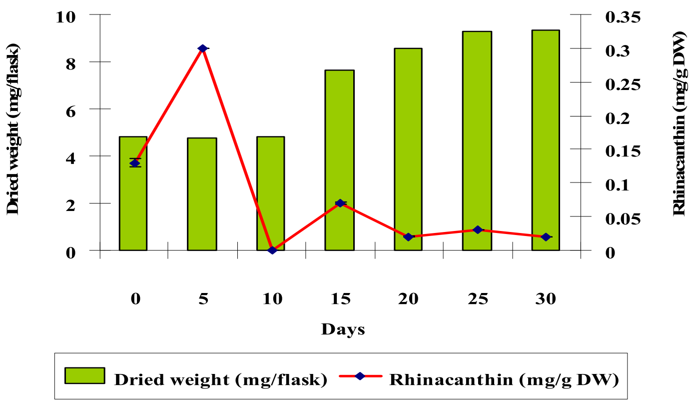


Figure 4.21 Time course of rhinacanthin-D production in *R. nasutus* root cultures

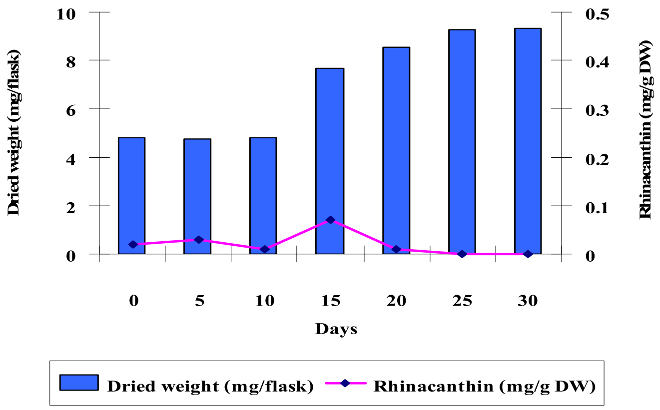


Figure 4.22 Time course of rhinacanthin-N production in *R. nasutus* root cultures

Table 4.12 Time course of rhinacanthin-C, -D and -N production in *R. nasutus* root cultures

Days	Rhinacanthin content (mg/g DW ± S.D.)		
	Rhinacanthin-C	**Rhinacanthin-D**	**Rhinacanthin-N**
0	1.60 ± 0.007	0.13 ± 0.007	0.02 ± 0.000
5	1.53 ± 0.030	0.03 ± 0.000	0.03 ± 0.000
10	0.62 ± 0.009	n.d.	0.01 ± 0.000
15	3.39 ± 0.040	0.07 ± 0.001	0.07 ± 0.000
20	1.15 ± 0.001	0.02 ± 0.001	0.01 ± 0.000
25	0.99 ± 0.007	0.03 ± 0.000	n.d.
30	0.72 ± 0.008	0.02 ± 0.000	n.d.

n.d. : can not calculated due to the area under the peak is under the lower limit of detection

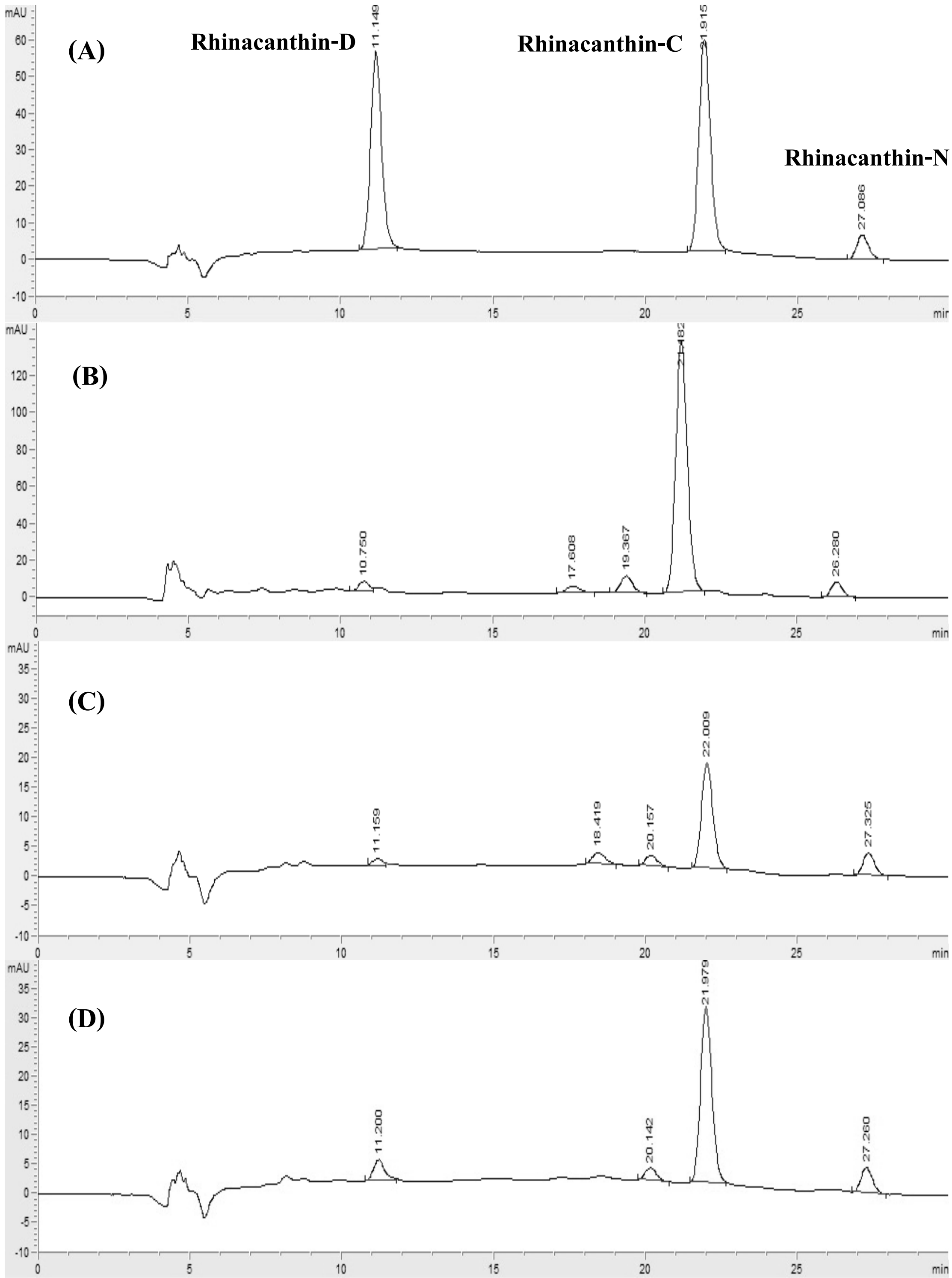


Figure 4.23 HPLC-chromatograms of the standard rhinacanthin (A); extracts of root cultures (B); extracts of intact root (C) and extracts of intact leaves (D)

Table 4.13 Rhinacanthin-C, -D and -N content in root cultures of *R. nasutus*, intact roots and intact leaves

Samples	Rhinacanthin content (mg/g DW ± S.D.)		
	Rhinacanthin-C	**Rhinacanthin-D**	**Rhinacanthin -N**
Root cultures (15 days old)	3.39 ± 0.040	0.07 ± 0.001	0.07 ± 0.000
Intact roots	8.18 ± 0.069	n.d.	0.63 ± 0.007
Intact leaves	12.86 ± 0.159	0.89 ± 0.009	0.69 ± 0.017

n.d. : can not calculated due to the area under the peak is under the lower limit of detection

CHAPTER 5

CONCLUSIONS

From this research work the following conclusions can be drawn:

1. Medium manipulation and elicitation techniques were not successfully induced rhinacanthin production in *R. nasutus* cell suspension cultures.

2. The whole leaf explants were appropriate for initiation of *R. nasutus* root cultures.

3. Both growth and rhinacanthin-C formation of *R. nasutus* cultures were inhibited by light.

4. *R. nasutus* root cultures were established in MS liquid medium supplied with 3.0 mg/l IBA and 30 g/l sucrose. The root cultures was capable of producing rhinacanthin-C, but in very low amount (0.03 ± 0.001 mg/g DW).

5. Improving of rhinacanthin production in *R. nasutus* cultured roots was achieved by culturing the root culture in the MS semisolid medium supplied with 3.0 mg/l IBA and 30 g/l sucrose.

6. Study on the time-course of growth of the *R. nasutus* root cultures showed that the root cultures spent a period of ten days for cell adaptation in a lag phase and showed long linear phase (15 days) before entering to the stationary phase.

7. Study on the time-course of rhinacanthin production in the *R. nasutus* root cultured showed that rhinacanthin production was found to increase with highest its rhinacanthin accumulation at the linear phase (day 15) of the growth cycle.

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